RND Proteins in *Staphylococcus aureus*: Their Function and Impact on Fitness, Resistance and Virulence

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Summary

Staphylococcus aureus is one of the leading causes of nosocomial and community-acquired infections. It is a frequent colonizer of the human body, but can also lead to life-threatening diseases. Due to the high adaptability of *S. aureus* to its environment and certain antibiotics, treatment of *S. aureus* infections has become increasingly difficult.

Antibiotic resistance can occur by several different mechanisms, thereof one is the unspecific export via multidrug efflux pumps as shown for AcrB in *Escherichia coli*. AcrB belongs to the resistance-nodulation-cell division (RND) family, which is conserved throughout all three kingdoms of life and has diverse biological functions. *S. aureus* possesses three uncharacterized RND proteins: the AcrB homologue SA2056, the MmpL-like SA2339 and SecDF. The aim of this project was to characterize RND proteins in *S. aureus* and to study their role in fitness, resistance and virulence.

Deletion of *sa2056* and *sa2339* had no influence on growth, fitness and resistance under the conditions tested. However, SA2056, encoded by the gene *sa2056* situated downstream of *femX*, was shown to interact with itself and with several other factors involved in cell wall synthesis: FemB and the penicillin binding proteins PBP1 and PBP2. FemX and FemB both play an important role during cell wall synthesis by catalyzing the addition of the first and last two glycines to the pentaglycine crossbridge, respectively. Interaction with FemB was confirmed by pull-down experiments. However, a *sa2056 femB* double mutant did not show any aggravation of the *femB* phenotype regarding β-lactam sensitivity and lysostaphin resistance. These results suggest an accessory role of SA2056 in the peptidoglycan synthesis pathway.

The third RND protein, SecDF, is part of the Sec secretion system and was shown to enhance protein secretion in *E. coli*. Deletion of *secDF* had a pleiotropic effect. Growth under normal conditions was only slightly reduced, which was exacerbated at 15 °C as shown for *E. coli* and *Bacillus subtilis secDF* mutants. Susceptibility towards β-lactam and glycopeptide antibiotics in an *S. aureus* methicillin susceptible and resistant background, as well as towards RND substrates, was increased. The ultrastructure of *secDF* mutant cells displayed a partly impaired cell division. Furthermore, autolysis was increased. Transcription and expression analysis of specific virulence determinants indicated further indirect effects on important processes, not only secretion.

A quantitative secretome analysis revealed an altered composition of the extracellular proteins in the *secDF* mutant with numerous Sec-dependent virulence factors decreased. The

diminished amounts of virulence determinants led to a significantly reduced adhesion, invasion and cytotoxicity of the *secDF* mutant in human umbilical vein endothelial cells and to a reduced pathogenicity using an insect infection model. Altogether, this study identified SecDF to be a promising therapeutic target for controlling *S. aureus* infections, since the absence of SecDF reduced both *S. aureus* virulence and resistance to well established antibiotics.

Zusammenfassung

Staphylococcus aureus ist einer der häufigsten Erreger von nosokomialen und nicht-nosokomialen Infektionen. Dieser opportunistische Erreger kann den Menschen asymptomatisch besiedeln, aber auch lebensbedrohliche Krankheiten hervorrufen. Die hohe Anpassungsfähigkeit gegenüber seiner Umwelt und gewisser Antibiotika, sowie ein breites Arsenal von Virulenzfaktoren tragen zum Erfolg dieses Pathogens bei.

Die Antibiotika Resistenz kann durch verschiedene Mechanismen entstehen, ein Mechanismus ist der unspezifische Export durch Efflux-Pumpen, wie bei AcrB in *Escherichia coli*. AcrB gehört zur so genannten „resistance-nodulation-cell divsion" (RND) Familie, welche in allen drei Reichen Archaeen, Prokaryoten und Eukaryoten vertreten ist. RND Proteine können unterschiedliche biologische Funktionen ausüben. *S. aureus* besitzt drei uncharakterisierte Proteine, welche aufgrund ihrer Aminosäurensequenz- und Struktur-Homologie zur RND Familie gehören: SA2056, SA2339 und SecDF. Das Ziel dieser Arbeit war die Charakterisierung dieser Protein-Gruppe und ihre Auswirkung auf Fitness, Resistenz and Virulenz in *S. aureus*.

Unter den getesteten Bedingungen, hatte die Deletion von *sa2056* und *sa2339* keinen Einfluss auf das Wachstum, die Fitness und Resistenz. Bei SA2056 konnte jedoch eine Interaktion mit sich selbst und anderen Faktoren, welche an der Zellwandsynthese beteiligt sind, nachgewiesen werden. Die Interaktion zwischen SA2056 und FemB wurde mit einem Pull-Down Experiment bestätigt. FemB ist an der Peptidoglykan (PG)-Vorläufersynthese beteiligt. Eine *sa2056 femB* Doppelmutante zeigte jedoch keine Verstärkung des *femB* Phänotyps bezüglich seiner β-Lactam Empfindlichkeit oder Lysostaphin Resistenz. Die Resultate weisen auf eine eher kleine Helfer-Rolle von SA2056 in der PG-Synthese hin.

Das dritte RND Protein SecDF ist ein Teil der wichtigen Sec Transportmaschinerie und steigert in *E. coli* den Export von Proteinen aus dem Zytoplasma. Die Deletion von *secDF* in *S. aureus* zeigte einen vielseitigen Effekt. Unter normalen Bedingungen war nur ein etwas vermindertes Wachstum sichtbar, welches sich bei 15 °C jedoch drastisch verstärkte. Einen ähnlichen kälteempfindlichen Phänotyp wurde bereits in *E. coli* und *Bacillus subtilis secDF* Mutanten nachgewiesen. Die Empfindlichkeit gegenüber β-Lactam und Glykopeptid Antibiotika in einem Methicillin empfindlichen und einem Methicillin resistenten Stamm sowie gegenüber RND-Substrate, war erhöht. Elektronenmikroskopische Aufnahmen der Zellen zeigte eine gestörte Zellteilung beim Fehlen von SecDF. Zudem wurde eine erhöhte Autolyseaktivität festgestellt.

Die Analyse der Expression und Transkription betroffener Virulenzfaktoren zeigte einen zusätzlichen, womöglich indirekten Effekt auf weitere Prozesse und nicht nur auf die Proteinsekretion.

Eine quantitative Sekretom-Analyse der *secDF* Mutante zeigte ein verändertes Exoproteom mit einer reduzierten Menge von Sec-abhängigen Virulenzfaktoren. Dies führte zu einer signifikant reduzierten Adhäsion an humanen Nabelschnur-Endothelzellen (HUVECs), und signifikant reduzierten Invasion und Zytotoxizität. Zudem war die Virulenz der *secDF* Mutante in einem *Galleria mellonella* Infektions-Model signifikant reduziert. Insgesamt konnte diese Arbeit zeigen, dass SecDF ein sehr interessantes Zielmolekül zur Bekämpfung dieses Erregers ist, da nicht nur die Virulenz, sondern auch die Resistenz gegen gut etablierte Antibiotika reduziert ist.

1 Introduction

1.1 *Staphylococcus aureus*

Staphylococcus aureus is a Gram-positive, non-motile bacterium and belongs to the family of micrococcaceae. Its name arises from the irregular grape like clustering of the cocci, as well as the yellow colouring of the colony forming units (Greek: staphyle – a bunch of grapes; Greek: kokkos – grain, seed; Latin: aurum – gold) (Figure 1). The cell size ranges from 0.8 to 1 µM in diameter. The genome of *S. aureus* has a size of approximately 2.8 mb and consists of 2'592-2'748 protein-coding open reading frames (ORF) depending on strain (94). The highly conserved core genome consists of around 78 % and comprises mainly housekeeping genes, the remaining 22 % are non-essential genes and mobile genetic elements (82, 157). The G+C-content is low and varies around 33 % (152). The first sequenced *S. aureus* genomes N315 and Mu50 were published in 2001 (152). Up to date there are 32 published whole genome sequences at the National Center for Biotechnology Information (NCBI) (190) and there are plenty of unfinished genomic sequence data available in the High Throughput Genomic (HTG) GenBank Division (210).

S. aureus colonizes the skin and mucous membrane of humans as well as several animals (167). About 60 % of the healthy human population is colonized intermittently, whereas 20 % of the population belongs to the persistent carriers (146). This is relevant since numerous reports point towards an association between *S. aureus* infections and *S. aureus* nasal carriage as a source of infection, moreover the nose is the most frequently colonized body part in humans (146, 290, 297, 299). *S. aureus* is an opportunistic pathogen that can cause a wide spectrum of diseases, ranging from superficial skin infections and food-poisoning to life-threatening endocarditis, osteomyelitis, septicaemia or toxic shock syndrome. In the USA a national wide study of healthcare-associated infections among surgical patients revealed *S. aureus* to be the most frequently found pathogen (36 %), along with coagulase-negative staphylococci (17 %), *Escherichia coli* (10 %) and enterococci (8 %). Eleven percent of the *S. aureus* isolates were methicillin resistant *S. aureus* (MRSA) (241). The increasing numbers of nosocomial and community-acquired (CA) MRSA infections are a great problem for healthcare facilities (68, 160, 174, 208).

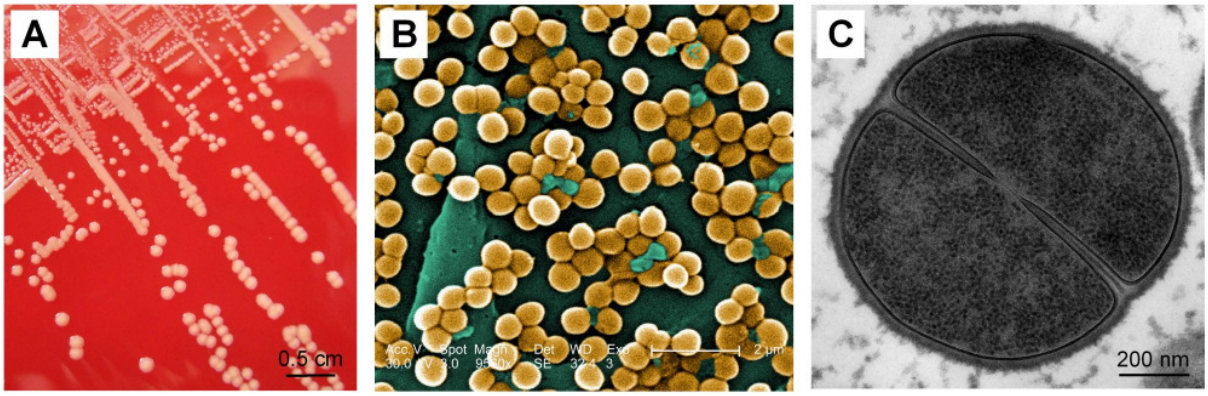

Figure 1. Images of *S. aureus*. (A) *S. aureus* colonies on sheep blood agar plates. (B) Scanning electron micrograph pictures of *S. aureus*. Picture was taken from (37). (C) Transmission electron micrograph of a separating *S. aureus* cell.

1.1.1 Cell wall

S. aureus is surrounded by a thick cell wall. The function of the bacterial cell wall is diverse; its main purpose is to protect the bacterium against the high internal osmotic pressure of 20 atm (178) and to maintain the cell shape. Thirty to 70 % of the cell wall of Gram-positive bacteria consists of multiple layers of cross-linked peptidoglycan (PG) (20-40 nm) with bound surface proteins and wall teichoic acids (242). The disaccharide sugar structures of the PG consists of alternating *N*-acetylglucosamine (Glc*N*Ac) and *N*-acetylmuramic acid (Mur*N*Ac), which are covalently linked by β-1,4 glycosidic bonds. The *S. aureus* stem pentapeptide, L-alanine-D-*iso*-glutamine-L-lysine-D-alanine-D-alanine, branches off the carboxyl group of the uridine-diphosphate (UDP)-Mur*N*Ac (242). The first biosynthetic steps are accomplished by the enzymes MurA to MurF and the D-alanine-D-alanine ligase Ddl (Figure 2). The transferase MraY anchors the soluble UDP-Mur*N*Ac pentapeptide to the undecaprenyl phosphate (C_{55}-P) yielding the membrane-bound intermediate lipid I (25). The lipid carrier C_{55}-P is needed for the transport of the hydrophilic PG-precursor across the membrane; furthermore it is also involved in other biosynthetic pathways, such as the teichoic acid synthesis (65). The glycosyl transferase MurG catalyzes the addition of Glc*N*Ac to the membrane linked Mur*N*Ac-pentapeptide resulting in lipid II (288). Still at the cytoplasmic side of the membrane, the three non-ribosomal peptidyltransferases FemABX add five glycines to the ε-amino group of the L-lysine in a sequential way. The PG precursor is transported across the membrane by a yet unknown mechanism (26) and the pentaglycine bridge is cross-linked to the adjacent stem peptide connecting the L-lysine to the D-alanine, thereby cleaving off the last D-alanine. The final steps of the PG polymerization, the transglycosylation and the transpeptidation, are catalyzed by the penicillin binding proteins (PBPs). Methicillin susceptible *S. aureus* possess

four PBPs; the high-molecular weight PBPs1-3 and the low-molecular weight carboxypeptidase PBP4 (118). PBP1, 3 and 4 are monofunctional and exhibit transpeptidase activity, only PBP2 is a bifunctional enzyme with transpeptidase and transglycosylase activity (93, 96, 184).

As the bacterial cell wall is important for both survival and virulence of microorganisms, it is an excellent target for antibiotics. The two main antibiotics of choice for treatment of *S. aureus* infections are cell wall active antibiotics: penicillinase-resistant β-lactams for MSSA- and vancomycin for MRSA-infections (229).

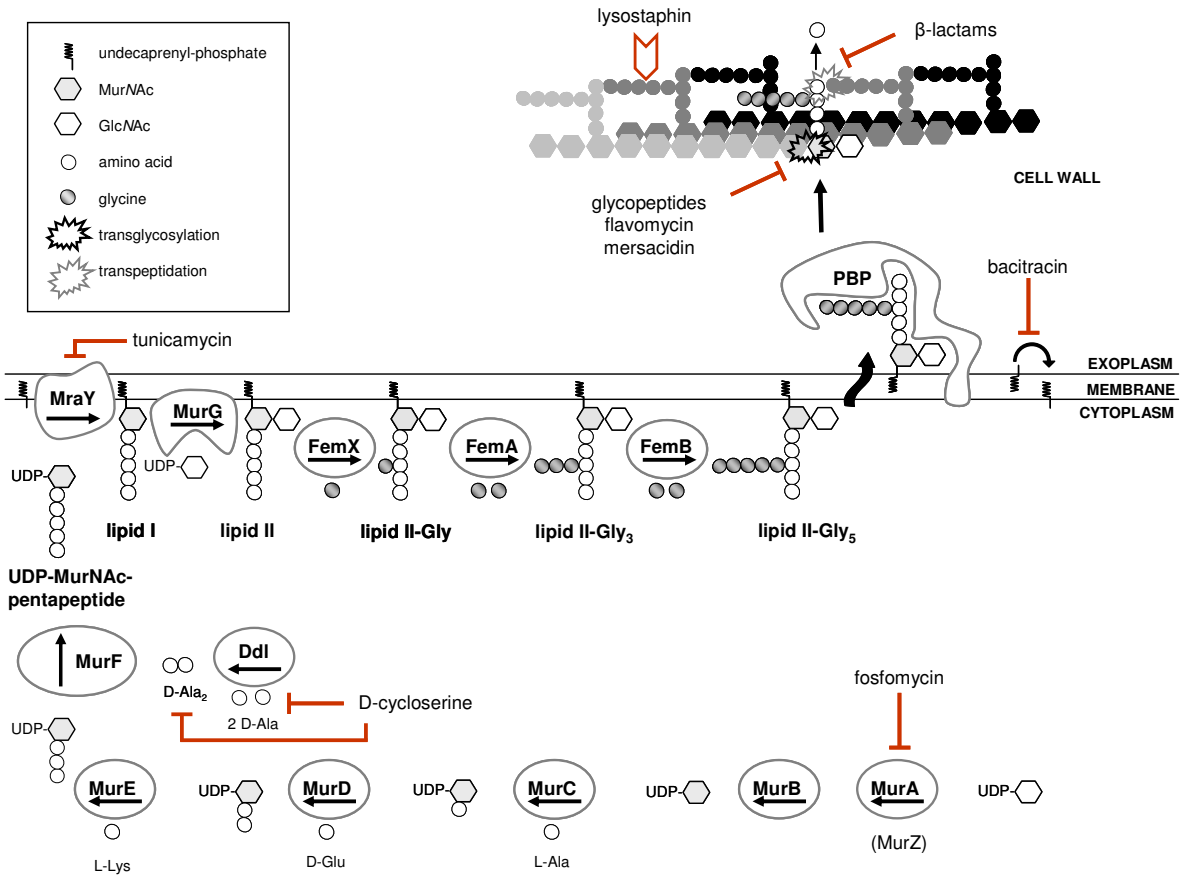

Figure 2. Schematic representation of the peptidoglycan biosynthesis pathway with the corresponding enzymes. The first steps of the PG synthesis take place in the cytoplasm. After the addition of the five depicted amino acids to the sugar component Mur*N*Ac, the Mur*N*Ac-pentapeptide is bound to the membrane by its attachment to the undecaprenyl phosphate. The second sugar moiety Glc*N*Ac is then added to the lipid I and the peptidyltransferases FemABX add five glycines to the L-lysine. On the outer side of the membrane the PBPs catalyze the cross-linking of the stem peptides by transglycosylation and transpeptidation. A selection of cell wall active antibiotics is indicated in red. Adapted from (166).

1.1.2 Antibiotic resistance

Penicillin was discovered by chance in 1928 by Alexander Fleming (83). *S. aureus* culture plates got accidentally contaminated by a mould, which displayed a growth inhibition zone. It took several years until the importance of this finding, as well as the skills to produce penicillin in large quantities, was realised. In the early 1940s penicillin was applied in clinical practice and penicillin mass-production started. Soon afterwards the first penicillin resistant *S. aureus* appeared, producing the β-lactam ring hydrolyzing enzyme penicillinase (12). Nowadays more than 95 % of staphylococcal isolates carry a penicillinase in North America (160). A second development of β-lactam resistance occurred in 1961, only one year after introducing the new semi-synthetic penicillinase resistant β-lactam antibiotic methicillin (133, 234). It took over 20 years to discover the responsible gene for methicillin resistance, *mecA* (111). Since the discovery of penicillin, more antibacterial agents have been discovered and developed, followed by a rapid evolution of antibiotic resistant bacteria.

Microorganisms employ three different strategies to conquer antibiotics; (i) drug modification or inactivation, (ii) drug target modification, (iii) enhanced efflux of drug (53). These alterations can be achieved, by acquiring resistance genes, by gene regulation or by chromosomal mutations; the spontaneous mutation frequency lies between 10^{-7} and 10^{-10}.

1.1.2.1 β-lactam resistance

β-lactams have structural similarities to the D-ala$_2$ end of the PG pentapeptide and can therefore bind irreversibly to the active site of the PBPs, thereby inhibiting their mode of action (93). This leads to reduced cross-linking, disturbance of the cell wall integrity, to cell stress response and eventually lysis and cell death (148). The precise mechanism is still unclear and is dependent on the drug concentration.

S. aureus has two main mechanisms to become β-lactam resistant: (i) By the production of the penicillinase BlaZ, which is able to inactivate penicillin by hydrolyzing the β-lactam ring or (ii) by the acquisition of an additional PBP; PBP2a (or PBP2'). The second mechnism displays a broader activity, since it also confers resistance against penicillinase resistant β-lactams.

PBP2a is encoded chromosomally by the *mecA* gene and displays a lower affinity to β-lactam antibiotics than the intrinsic PBPs 1-4 (111, 231). Together with the remaining transglycosylation activity of PBP2 it completes the cross-linking of the PG in presence of β-lactams (224).

mecA is located on a large mobile genetic element, the staphylococcal cassette chromosome *mec* (SCC*mec*) (128, 142). The SCC*mec* integrates at a specific integration site sequence (ISS) at the 3'-end of *orfX*. *orfX* has recently been described to encode a RlmH type ribosomal methyltransferase, which methylates the 70 S ribosomes (27). Transcription and expression of *orfX* is not disturbed by the incorporation of SCC*mec*. The origin of the SCC*mec* element still remains unclear, but it is proposed to derive from animal associated coagulase negative staphylococci (280). The International Working Group on the Classification of Staphylococcal Cassette Chromosome Elements (129) was established to standardize and simplify the classification of these mobile elements. SCC*mec* elements should exhibit the following four characteristics: (i) contain the *mec* gene complex including *mecA*, (ii) contain the *ccr* gene complex with *ccrAB* and/or *ccrC*, (iii) integrate at a specific integration site, the ISS and (iv) possess flanking direct repeat sequences containing the ISS. To date there are eleven SCC*mec* types, which are defined by point (i) and (ii). The size of SCC*mec* varies between 21-67 kb depending on the elements (119). Besides *mecA*, the *mec* gene complex comprises the entire or truncated regulatory genes *mecI* and *mecR1* and the *ccr* gene complex, which holds the recombination and excision locus. The non-essential components of the resistance island are called junkyard or joining regions (J regions). They can be used for subtyping and are a hotspot for integration of additional resistance genes.

Furthermore, there are borderline resistant *S. aureus* strains, which display a low level methicillin resistance without the production of PBP2a (181). The reduced susceptibility against penicillinase-resistant penicillins was first thought to be caused by the β-lactamase hyperproduction (170), but according to Montanari *et al.* borderline resistant strains produce, in addition to the penicillinase, a methicillin specific β-lactamase (methicillinase), which is responsible for the reduced susceptibility (164, 180). Another possibility are amino acid (aa) substitutions in the PBPs, which result in a modified drug reactivity (103, 188, 273).

1.1.2.2 Regulation of β-lactam resistance

Both β-lactam resistance mechanisms *blaZ* and *mecA* are regulated by a cognate two- and three-component system BlaR1-BlaI and MecR1-MecI-MecR2, respectively (as reviewed in (90)). Most studies were performed with the *blaZ* two-component system (TCS).

Regulation of *blaZ*

The *blaZ* regulating TCS is usually plasmid borne (102, 237, 238) and composed of the structural gene *blaZ*, the sensor transducer *blaR1*, as well as the repressor *blaI*. *blaZ* and

blaR1-blaI are divergently transcribed and have an overlapping promoter/operator region (Figure 3). β-lactam antibiotics induce *blaZ* transcription by acylation of the active-site serine of the exoplasmic penicillin-binding domain of BlaR1 (298), which results in a conformational change and autocatalytic cleavage of the sensor transducer within the cytoplasmic domain (302). The so far described BlaR1 autoproteolytic activity seems to occur also without the induction by β-lactams, as shown recently in a heterologous expression system in *E. coli* (158). Following the metalloproteolytic cleavage of the dimeric repressor $BlaI_2$, which occurs directly by BlaR1 (158), BlaI is released from the promoter/operator region and the transcription of the *blaZ* and the *blaR1-blaI* operon is activated (302). BlaR1 is constantly replenished to ensure continuous cleavage of $BlaI_2$. As soon as the β-lactam concentration decreases, the autoproteolytic cleavage of the sensor transducer is reduced and the repressor is able to dimerize and block the promoter/operator region again.

Interestingly, an additional factor BlaR2, which is unlinked to the penicillinase plasmid, was proposed to be involved in *blaZ* regulation, but the precise function and role of BlaR2 was never elucidated (51).

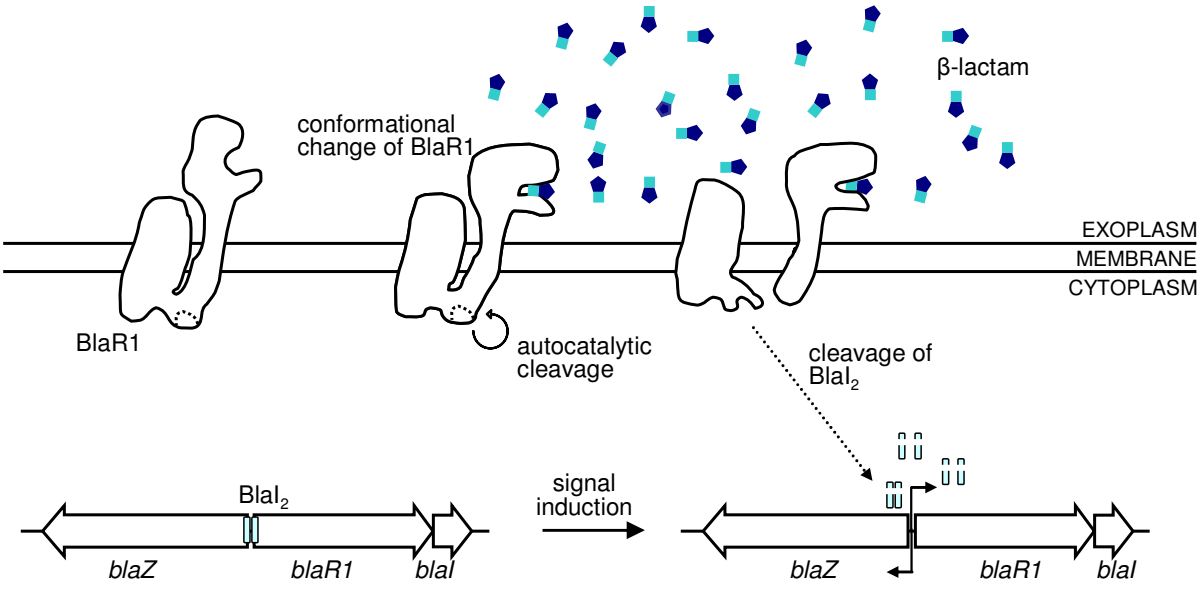

Figure 3. Regulation of β-lactam resistance by the two-component system BlaR1-BlaI. During the uninduced state, the homodimer $BlaI_2$ binds to the promoter/operator region of *blaZ* and the *blaR1-blaI* operon, preventing transcription of *blaZ*. After exposure to β-lactam, the antibiotic binds to the sensor domain of *blaR1* and autoproteolytic cleavage of BlaR1 is induced. This leads to the direct cleavage of $BlaI_2$ and induction of *blaZ* and the *blaR1-blaI* operon. Adapted and modified from (54).

Regulation of *mecA*

Due to strong homology, *mecA* regulation was originally thought to function similarly to the cognate *blaZ* TCS. But different induction kinetics of BlaR1 and MecR1 by β-lactams (172) and the recent finding of a third component, the anti-repressor *mecR2* lying downstream of *mecI* (6), indicate subtle differences between the two systems. *mecR2* is co-transcribed with the *mecR1-mecI* operon and MecR2 shows 60-64 % aa identity with the XylR repressor of the xylose operon of *S. xylosus* (6).

The revised model is as followed: β-lactams induce the signal transduction cascade by a yet unknown mechanism by binding to the sensor domain of MecR1 (163). Activation leads to the transcription of *mecA* and the regulatory operon *mecR1-mecI-mecR2*. MecR2 supports the inactivation of the repressor MecI dimers by proteolytic cleavage (6). Furthermore, $MecI_2$ proteolysis was shown to be crucial for the optimal expression of the β-lactam resistant phenotype (7).

Due to the high aa sequence identity (61 %) of MecI and BlaI (91), both repressors are able to bind to either promoter/operator region of *mecA-mecR1-mecI* and *blaZ-blaR1-blaI*. In contrast to the co-repression, cleavage of the repressor is only achieved by their cognate sensor transducers MecR1 and BlaR1, which have an aa sequence identity of 34 % (172, 298).

1.1.2.3 Chromosomal factors influencing β-lactam resistance

The level of β-lactam resistance does not directly correlate with the amount of PBP2a, moreover, it was shown that several chromosomally encoded factors influence β-lactam resistance (38, 110, 185). These genes were initially called factors essential for methicillin resistant (*fem*) or auxiliary (*aux*) factors (16, 62). The majority is directly or indirectly involved in cell wall metabolism. However, also genes of the stress response, of ABC transporters or protein kinases were found to influence methicillin resistance (16, 17, 62, 63, 251, 300).

1.1.2.4 Glycopeptide resistance

Vancomycin is one of the most clinically relevant glycopeptides and was discovered in the early 1950s. Vancomycin binds to the acyl-D-alanyl-D-alanine of the pentapeptide and prevents transpeptidation and transglycosylation of the PG precursors, the latter due to steric hindrance (13, 230). Widespread use only began 30 years after it was discovered (144). This was primarily due to the simultaneous development of 2nd and 3rd generation β-lactam

antibiotics, as well as early problems with vancomycin toxicity, which was later found to be caused by impurities in the preparations (98). The increasing appearance of MRSA in the 1980s led to an increased use of vancomycin as an antibiotic of last resort. First vancomycin resistant microorganisms were reported in *Enterococcus faecium* in 1986 (154) and in the coagulase-negative *Staphylococcus haemolyticus* in 1987 (248). Ten years later the first MRSA with reduced susceptibility to vancomycin was described in Japan (120). Two different vancomycin resistance strategies have evolved in the past decades, which are also reflected in the MIC of the isolates. The vancomycin intermediate resistant *S. aureus* (VISA) has an MIC of 4-8 µg/ml and the high-level vancomycin resistant *S. aureus* (VRSA) has an MIC of >16 µg/ml. VISA display a thickened and/or poorly cross-linked cell wall with more free D-alanyl-D-alanine residues that are available to bind to vancomycin. Furthermore a "clogging-phenomenon" was observed by vancomycin molecules trapped in the PG meshwork, preventing the other molecules to reach the actual site of PG biosynthesis. Increased cell wall thickness is achieved by decreased cell wall turn over and reduced autolytic activity or increased cell wall synthesis. Therefore, it is often accompanied by a high fitness cost and an unstable resistance phenotype, which is frequently lost when passaged without vancomycin. The reduced vancomycin susceptibility is a result of point mutations in regulatory genes (21, 56, 122, 187, 191, 247, 251). In 2002 the first high-level VRSA was identified with an MIC of > 32 µg/ml in the USA (39). This clinical isolate carried a multiresistance conjugative plasmid harbouring the *vanA* gene cluster mediated by transposon Tn*1546* (296). From the same site of infection a vancomycin resistant *Enterococcus faecalis* (VRE) was isolated carrying the identical *vanA* gene cluster on a different conjugative plasmid. This suggests that one or two genetic events took place: interspecies transduction of the Tn*1546* or conjugative transfer of the VRE plasmid, excision and integration of Tn*1546* in another plasmid. So far, no dissemination of VRSA from person-to-person has been observed (257).

To date, eight different Van phenotypes have been described in enterococci, but only the VanA resistance type has been observed in *S. aureus* (36). The VanA gene cluster consists of the two regulatory genes *vanRS* and the five resistance genes *vanHAXYZ*, whereof *vanHAX* are essential for vancomycin resistance. These enzymes ensure the substitution of the PG-pentapeptide D-ala-D-ala termini into D-ala-D-lac depsipeptide, which displays a 1000 times lower affinity to vancomycin (30). Vancomycin resistance is inducible via the sensor histidine-kinase VanS and the response regulator VanR. The fitness cost of vancomycin

resistance in the presence of the antibiotic is high, whereas during the un-induced state, there is only a slight burden, possibly due to low level expression of the *van* genes (87).

1.1.3 Virulence factors

The success of *S. aureus* is not only based on its ability to adapt to the environment, but also on its arsenal of virulence factors, which can cause a wide range of infections (5). They are roughly divided into two groups: surface-associated and secreted virulence factors. The former mainly comprises factors, which are important during the early phases of an infection and promote colonization and evasion of the host immune system (160). The latter is important at later stages of an infection and facilitates invasion and dissemination of the bacteria as well as the acquisition of nutrients. The following chapter introduces a selection of important virulence determinants in *S. aureus*.

1.1.3.1 Surface-associated virulence factors

Many surface-associated virulence factors belong to the microbial surface components recognizing adhesive matrix molecules (MSCRAMM) and are covalently attached to the cell wall (50). Proteins destined for the cell wall contain a C-terminal LPXTG-motif, which is recognized and cleaved by the sortase A (SrtA) (165, 244, 246). Surface proteins containing a signal peptide with an YSIRK/GS motif are addressed to the cross wall near the division site and eventually across the whole cell surface, whereas proteins without the YSIRK/GS motif are trafficked to the cell poles (64).
One of the key functions of the MSCRAMMs is to mediate adhesion to the extracellular matrix and host proteins, such as fibronectin, fibrinogen, collagen, elastin and vitronectin.

Fibronectin binding proteins (FnBP)

S. aureus has two fibronectin binding proteins A and B (FnBPAB). Depending on the region they share 45 to 95 % aa identity (139, 258). Both FnBPs are able to bind fibronectin, elastin and platelets (112, 293) and they were shown to act as invasins in endothelial, epithelial and fibroblast cells by binding to the integrin $\alpha_5\beta_1$ host cell receptor via fibronectin (259, 260). The uptake of *S. aureus* by endothelial cells is believed to promote bacterial dissemination and persistence (261). Most clinical isolates harbour at least one FnBP (219). FnBPA, but not FnBPB, binds to fibrinogen and can induce platelet aggregation (114), thus the two proteins are not entirely redundant.

S. aureus strain Newman possesses two truncated FnBPs, that lack the LPXTG-motif due to a point mutation resulting in an early stop codon and are entirely secreted (100).

Clumping factor (Clf)

The two structurally similar clumping factors ClfA and ClfB belong to the serine-aspartate repeat (Sdr) family of covalently attached surface proteins (49, 141, 168). Both proteins are fibrinogen binding proteins that promote cell clumping and platelet aggregation (168, 204) and thereby contribute to endovascular infections (81, 182). In addition, ClfB supports binding to squamous epithelial cells and keratinocytes, most likely via cytokeratin 10, which is exposed on both cell types (205, 291). Furthermore, a human nasal colonization study revealed ClfB to be important for *S. aureus* persistence (297).

ClfA plays a role in the host immune evasion by binding the host complement control protein, factor I (107). The highly potent opsonin C3b, which is also part of the complement system, marks *S. aureus* cells for phagocytosis and can be inactivated by the regulatory protein factor I (57). By hijacking factor I, *S. aureus* increases C3b cleavage and thereby decreases phagocytosis. In addition, binding of fibrinogen to ClfA is proposed to be necessary for factor I activation (106)

Protein A (SpA)

The cell wall anchored protein A (SpA), encoded by the gene *spa*, is particularly known for its immunomodulatory effects by binding with high affinity to the Fc region of immunoglobulin G (IgG), thereby lowering phagocytosis (85, 179, 221). Recently, it was also shown to bind and activate the tumor necrosis factor α (TNFα) receptor 1 (TNFR1) (97) and trigger T cell-independent B cell proliferation (14). Further adhesive properties are the binding to the von Willebrand factor (vWF), which is a large glycoprotein mediating platelet adhesion at sites of vascular injury (109) as well as to the platelet receptor gC1qR/p33 (192). The complex role of SpA has led to several studies addressing the question to what extent SpA contributes to the virulence of *S. aureus*, which is still being debated. However, SpA seems to play a crucial role in biofilm related subcutaneous catheter infections in mice (176) and a SpA-deficient strain showed decreased virulence in a murine septic arthritis model (215).

Other prominent MSCRAMMs are the collagen-binding protein (Cna), the serine-rich adhesin for platelets protein (SraP), the Iron-regulated surface determinant (Isd) protein family and the Sdr family. For more details see reviews (50, 112).
A second group of adhesins are the secretable expanded repertoire adhesive molecules termed SERAM (43). The group characterizes secreted and non-covalently surface-associated proteins.

Coagulase (Coa)

The fibrinogen binding protein Coa is secreted into the media, however, it has also been found to remain partly surface-associated (23, 169). Coa promotes clotting of plasma or blood by binding to prothrombin and converting it non-enzymatically into its active form, thereby inducing polymerization of fibrinogen to fibrin (23, 89). Furthermore, Coa was shown to adhere to immobilized platelets probably via fibrinogen (113). A second coagulase, the von Willebrand factor binding protein (vWbp), also promotes fibrin coagulation by prothrombin activation (149). It was shown that staphylococcal binding to fibrinogen or fibrin via Coa and vWbp is associated with abscess formation, bacterial persistence in host tissue and the escaping of the host immune system (45, 46, 101). So far, no conclusive role in pathogenicity can be ascribed to Coa, since several studies have shown a diverse effect (8, 46, 182, 222, 240, 266).

Extracellular adhesive protein (Eap/Map/P70)

The conserved adhesin Eap has a broad binding spectrum, which ranges from plasma and matrix glycoproteins to eukaryotic cell surfaces and staphylococcal cells (84, 126, 138, 171, 212). The rebinding to *S. aureus* cells is ascribed to the interaction between Eap and the neutral phosphatase (84). Moreover, it was found to enhance internalization of *S. aureus* into fibroblasts, epithelial cells and keratinocytes (31, 105). Eap was also shown to have immunomodulatory/anti-inflammatory properties by binding to the intercellular adhesion molecule 1 receptor (ICAM-1), thereby inhibiting neutrophil binding to endothelial cells and decreasing endothelial transmigration of neutrophils and their extravasation (104). By triggering TNFα release it promotes attachment to endothelial cells via SpA and the gC1qR/p33 receptor on endothelial cells (79).

Further well-known fibrinogen binding proteins belonging to the SERAM group are the extracellular fibrinogen binding protein (Efb/Fib) (213, 214), the fibrinogen binding protein A

(FbpA) (47) and the extracellular matrix binding protein (Emp), which displays a broad range of binding activity, including fibronectin, collagen and vitronectin (125).

In addition, there are several non-proteinaceous virulence factors, which play a role in host colonization or immune evasion, such as cell wall components including teichoic acids (88, 294, 295), capsular polysaccharides (207) and the polysaccharide intercellular adhesin (PIA) encoded by the *icaABCD* operon (206).

1.1.3.2 Secreted virulence factors

S. aureus secretes a variety of diverse virulence determinants, such as membrane damaging toxins, exfoliative toxins, superantigens, exoenzmyes and proteases. Probably the most prominent haemolytic toxin is the α-haemolysin, encoded in the *hla* gene. Hla is expressed in most strains and is a pore-forming monomeric toxin exhibiting not only haemolytic, but also dermonecrotic and neurotoxic properties (19, 71). The α-toxin monomer binds to the target cell, integrates into the cell membrane and oligomerizes to a cylindrical heptamer pore (265). This leads to uncontrolled ion efflux/influx and finally to rupture of the cell. β-haemolysin, the antagonist of Hla, is a sphingomyelinase C and hydrolyzes membrane phospholipids. Hlb displays a hot-cold phenotype, leading to a stronger lysis after an incubation step at 4 °C (262). A third haemolysin, the δ-toxin, is encoded within the RNAIII molecule of the accessory gene regulator (*agr*) regulatory system (10). Hld belongs to the amphipathic peptide group of phenol-soluble modulins (PSM), which have diverse functions in pathogenicity (220, 292). Furthermore, there are the bi-component toxins, γ-haemolysin encoded by *hlgABC* and the Panton-Valentine leukocidin (PVL), encoded by *lukFS*. The γ-toxin is able to lyse erythrocytes and both toxins the γ-haemolysin and PVL affect neutrophils and macrophages. PVL, which was shown to be only produced by 2-3 % of the strains (150), has become more prominent in CA-MRSA, probably accounting for the increased virulence in those strains (69, 95, 275).

To manipulate the host immune system *S. aureus* possesses a number of superantigens. These polypeptides include several staphylococcal enterotoxins (SEA, SEB, SEC, SED, SEE and SEH) and the toxic shock syndrome toxin-1 (TSST-1). These exotoxins trigger excessive and abnormal T-cell activation by binding directly to the MHC class II molecules and the T-cell antigen receptor. This leads to a massive release of pro-inflammatory cytokines (TNFα and

interferon-γ) and further recruitment of B- and T-cells to the site of infection, which may eventually end in shock (159, 284).
Additional virulence factors affecting the host immunity are the chemotaxis inhibitory protein (CHIPS), the staphylococcal complement inhibitory protein (SCIN) and the formyl peptide receptor-like 1 inhibitory protein (FLIPr).

A further group of exoproteins are enzymes, which consist of proteases, lipases, hyaluronidases and nucleases. They are important for invasion and nutrient acquisition.

1.1.3.3 Regulation of virulence factors

It is crucial for the bacteria to coordinate the expression of the virulence factors during different growth phases, as well as in response to endogenous, host and environmental signals, such as cell density, energy availability, pH or CO_2. The regulation of virulence factors is very complex and controlled by several two-component regulatory systems, whereof *agr* (203) belongs to the best described global regulator in *S. aureus*. The *agr* system is growth phase dependent and consists of two divergent transcripts containing the *agrACDB* operon and RNAIII, which are under the control of the two promoters P2 and P3, respectively (Figure 4) (203). The RNAIII transcript also encodes the δ-haemolysin, which does not play a role in regulation (131). The integral membrane protein AgrB is responsible for the posttranslational processing of the in *agrD* encoded propeptide and its secretion (134, 135). The autoinducing peptide (AIP) consists of seven to nine aa's containing a pentapeptide thiolactone macrocycle and is recognized by the histidine kinase receptor of the membrane protein AgrC. Up to date there are four different *agr* subtypes, which express distinct AIPs, which vary in their aa composition (132, 134). The otherwise conserved *agr* locus contains a sequence variation spanning the section from the C-terminal region of *agrB* to the N-terminal region of *agrC* (Figure 4), which contains the locus for the peptide processing and ligand-receptor interactions (134). Consequently, the different AIPs are able to bind to the receptor of AgrC, but only the cognate signal peptide triggers the autophosphorylation of AgrC and the activation of the *agr* response. The binding of extrinsic AIPs leads to an intra-species cross-inhibition of *agr*-dependent virulence factor expression. The activated response regulator AgrA binds to the promoter region of P2 and P3 (147) and induces transcription of the RNAII and RNAIII transcripts, thereby completing the autoinduction cycle. The effector molecule RNAIII initiates the transcription of its target genes either directly or indirectly via other regulators (173, 243, 270). *in vitro* in the idealized batch culture, RNAIII upregulates most of

the secreted proteins, which are important for invasion and dissemination during the late exponential and stationary phase, and downregulates most of the surface proteins, which are central for colonization and evasion of the host immune system during the lag and early exponential phase (203). Since many studies were performed *in vitro*, the *agr* signalling during infections still remains poorly understood (48, 92, 227).

Additional to *agr*, there are many other virulence regulators, such as the SarA family, SaeRS, ArlRS, SrrAB and SvrA (as reviewed in (201)).

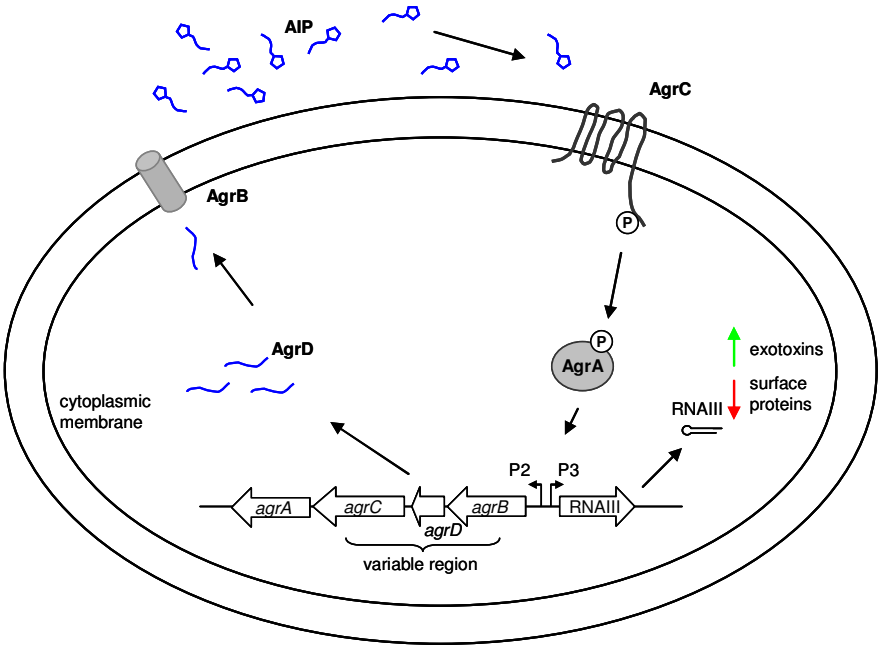

Figure 4. Schemativ overview of the *agr* quorum sensing system in *S. aureus*. *agrD* encodes the propeptide, which is subsequently processed to AIP and secreted by AgrB. AIPs bind and activate the sensor histidine kinase AgrC, which activates the effector AgrA. AgrA binds to the promoter region and induces transcription of the *agrACDB* operon and the major effector RNAIII leading to a growth phase dependent expression of surface and secreted proteins. Adapted from (202).

1.2 Protein secretion – "the pathways to pathogenesis" (254)

To fulfil their functions, the numerous virulence factors and antibiotic resistance determinants of *S. aureus* have to be transported across the membrane to their destined location, the cell membrane, the cell wall or the extracellular space.

Different secretion systems have evolved and were first described in Gram-negative bacteria. Up to date, there are six main secretion systems in Gram-negative bacteria: type I - type VI (Figure 5A). Substrates from the type I, III, IV and VI secretion systems are generally transported in one step and substrates from the type II and V secretion systems in two, relying on the Sec or Tat pathway for the precedent transport into the periplasm (279). The different secretion systems transport various substrates across the membranes, ranging from virulence factors and effector proteins to nucleic acids or flagellar proteins (279). Since the classification was originally introduced for Gram-negative bacteria possessing a cytoplasmic and an outer membrane, it is unfavourable to assign the transport systems of Gram-positive bacteria to the different type secretion systems of Gram-negative bacteria (67).

In Gram-positive bacteria the main pathway for protein export is the Sec secretion system. Other secretion or special purpose pathways are the twin-arginine (Tat) pathway, the Esx secretion system, ATP-bindinc cassette (ABC) transporters, the pseudopilin (Com) export pathway, holins and lysis (Figure 5B) (67, 254, 272). In addition, there are various assembly pathways, for example the pilus assembly in *Bacillus cereus* (29) or the flagella export apparatus in *Listeria monocytogenes* (20), which will not be further discussed (67, 245).

The Tat and the Sec secretion systems are ubiquitous in all three major kingdoms of life, but only the Sec system is essential for viability (1, 216). The Sec system is responsible for the export of most proteins and is best described in the Gram-negative model organism *E. coli* (as reviewed in (73, 74, 161)).

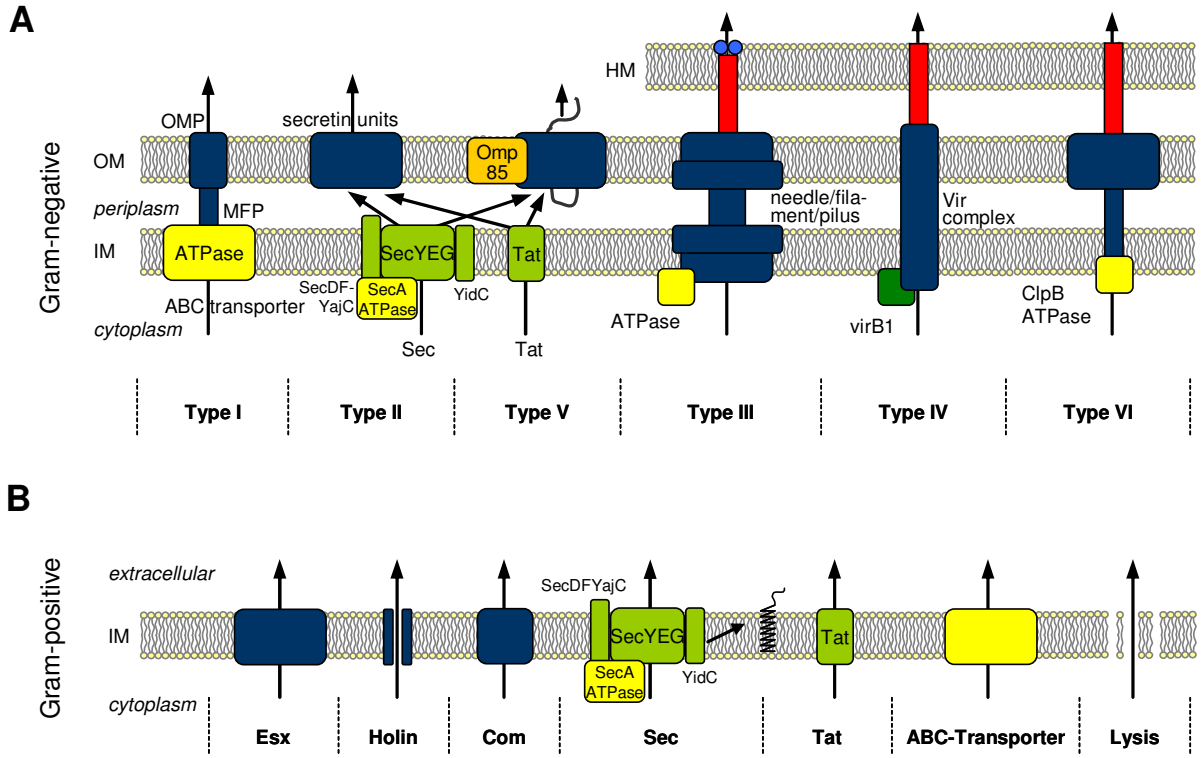

Figure 5. A simplified overview of bacterial secretion systems. Several different transport systems have evolved in microorganisms. (A) Type I - VI secretion systems in Gram-negative bacteria. (B) Secretion systems in Gram-positive bacteria. HM, host membrane; OM, outer membrane; IM, inner membrane; OMP, outer membrane protein; MFP; membrane fusion protein; ATPases and chaperones are shown in yellow; Esx, Esat-6 secretion system or WXG100 secretion system; Com, pseudopilin export pathway. Adapted and modified from (279).

Overview of the Sec pathway in *E. coli*

The Sec machinery consists of the protein conducting channel SecYEG with a single SecY molecule as the actual channel protein, which is clamped together by the SecE subunit (15, 217). The hourglass-like hydrophilic pore is blocked by a helix plug from the exterior, which is displaced for polypeptide translocation (15, 28). The exact function of SecG is still unknown, it is not essential for protein export or cell viability, but was shown to enhance translocation efficiency *in vitro* (108, 197, 198). SecG undergoes topology inversions coupled with the cycling of multiple molecules of SecA (183, 189, 199, 267). The motor protein SecA is involved in targeting of the preproteins to the translocase and is able to bind with high affinity to the SecYEG and with low affinity to acidic phospholipids (116). The conformational changes of SecA induced by binding and hydrolysis of ATP promotes protein translocation and is stabilized by the accessory complex SecDF-YajC (77, 78). Recently it was shown, that SecA alone is sufficient for translocation and ion channel activity in liposomes, but requires SecYEG and SecDF-YajC for specific and efficient transportation

(123, 124). The auxiliary membrane protein SecDF, which belongs to the resistance-nodulation-cell division (RND) family, forms a heterotrimeric complex with YajC and was shown to associate with SecYEG and YidC (76, 200), possibly as the linking molecule during Sec-dependent insertion of membrane proteins. It was furthermore shown that SecDF enhances protein translocation by binding to the preproteins and preventing backsliding by conformational changes of the head domain P1 (282). This last step is driven by the proton motive force (PMF) and is ATP-independent. YajC, which function is still unknown, was found to co-crystallize with the well known multidrug exporter AcrB (225, 269, 274).

Proteins containing an N-terminal signal peptide (SP) or a hydrophobic transmembrane (TM) domain are targeted to the translocase with the help of chaperones, which also prevent premature folding of the preproteins (41). There are two major targeting routes to the Sec translocase: The co- and the post-translational route (Figure 6) (74). The post-translational export is mainly used by secretory proteins, which together with membrane proteins containing large hydrophilic loops use the SecA cycling as driving energy (Figure 6A). Membrane proteins are mainly targeted as ribosome-bound nascent chain by the signal recognition particle (SRP, Figure 6B) and transported to the membrane receptor FtsY (153, 283). Upon GTP hydrolysis the ribosome-bound nascent chain is transferred to the translocon and co-translationally exported powered by the translating ribosome. Furthermore, small membrane proteins can be inserted Sec-independently by YidC (200) (Figure 6C). The signal sequence is cleaved off by the SPase I (59) and the preprotein is folded into its mature form with the help of different chaperones (2) or transported for further trafficking.

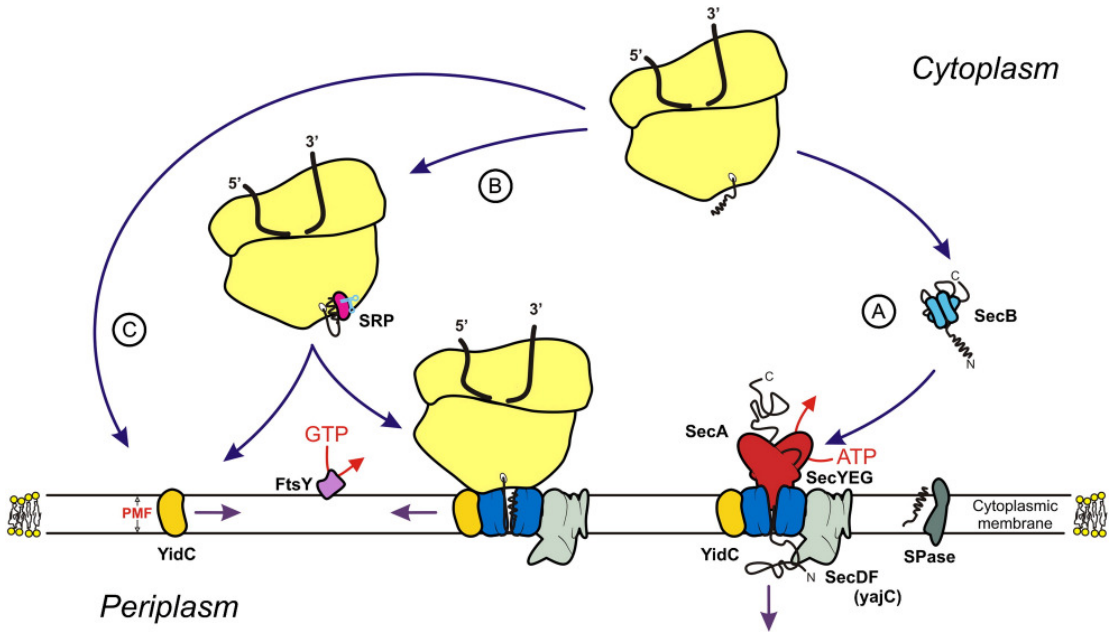

Figure 6. Schematic overview of the Sec pathway in *E. coli* with its different targeting routes. (A) Secretory preproteins are mainly post-translationally directed to the SecYEG translocation channel by SecB, a molecular chaperone, which is only found in Gram-negatives. The signal sequence is cleaved off by the signal peptidase (dark grey) at the outer side of the membrane. (B) Membrane proteins and some preproteins are co-translationally targeted as ribosome-bound nascent chain to the protein conducting channel by the SRP (pink). The SRP interacts with the N-terminal signal sequence or the hydrophobic TM segment of the nascent peptide. (C) Some membrane proteins are inserted into the membrane independently of the Sec system by YidC (dark yellow). Taken from (74).

The Sec pathway in *S. aureus*

Data on the Sec system in *S. aureus* is scarce. In different screens for essential genes *secY* was shown to be essential, whereas the results for *secDF*, *secE* and *secA* were ambiguous (9, 42, 86, 136). A *secG* deletion mutant showed an altered exoproteome, which was exacerbated in a *secG secY2* double mutant (253), however, the *secY2* single mutant did not show any effects on protein secretion. *secY2* together with *secA2* belong to the accessory Sec system, which is responsible for the export of the serine-rich adhesin for platelets protein (SraP) (255). The different *sec* mutants; *secG*, *secY2* and the double mutant *secG secY2*, had no effect on virulence in a mouse infection model (253).

SecDF was identified in a proteomic approach of membrane vesicles (155). Furthermore, its expression was slightly higher in a COL *sigB* deletion mutant (115).

Other secretion pathways in *S. aureus*

In addition to the general Sec pathway, *S. aureus* possesses more specified or special purpose secretion systems, which are responsible for the transport of a small number of proteins. The following section gives an overview of pathways found in *S. aureus*.

The **Tat system** in *S. aureus* is composed of the minimal translocase TatA and TatC (301). In contrast to the Sec system, Tat-dependent proteins are translocated in a fully folded form (18). So far, only the iron-dependent peroxidase FepB was found to have a typical twin-arginine (RR) signal peptide and to be exported by the Tat pathway (22). TatAC of *S. aureus* expressed in *B. subtilis* was shown to be intrinsically salt-sensitive (287).

Another transport system is the **Esx system**, which is also called the Esat-6, the WXG100 or the type VII secretion system following the secretion system nomenclature of Gram-negative bacteria (67). Esx is unique to Gram-positive bacteria and was first identified in *Mycobacterium tuberculosis* (70). In *S. aureus*, the Esx system is composed of a cluster with at least eight genes. The size of its target-proteins is approximately 100 residues and they exhibit a conserved Trp-X-Gly motif (WXG100 family proteins) (211). To date, the staphylococcal proteins EsxA, EsxB and the non-WXG100 motif protein EsaC were shown to be transported by the Esx system and the proteins EssABC and EsaD to be important for secretion (4, 32, 33, 44). The role of the secreted proteins EsxAB and EsaC is still unclear, but they were shown to play a role in *S. aureus* persistence and murine abscess formation (32, 33). The crystal structure of the integral membrane protein EssB was recently characterized and revealed a dimeric formation with a similar structure as serine/threonine protein kinases (303).

The **Com pathway** is involved in DNA binding and uptake and plays a role in natural competence in *B. subtilis* (75). In *S. aureus*, the pseudopilin pathway is partly represented with the four proteins ComC, ComGA, ComGB and GomGC (missing ComGD, ComGE and ComGG) (254). ComGC was shown to localize to the membrane, the cell wall and the cell surface (286). The biogenesis of ComGC relies on the stabilization activities of the thiol-disulfide oxidoreductase DsbA, as well as the cleavage by the signal peptidase ComC into its mature form. The precise role of the Com pathway in *S. aureus* is still unclear.

ABC transporters are composed of two transmembrane domains and two cytoplasmic nucleotide-binding domains with ATPase activity (61). *in silico* screening of the staphylococcal genome has identified numerous putative ABC transporters (209). They are involved in the secretion or uptake of a variety of molecules and exhibit diverse functions, which range from: (i) antibiotic resistance by serving as efflux pumps as proposed for the plasmid-encoded *vgaB*, which confers resistance to streptogramin A (3); (ii) the uptake of essential solutes, as suggested for the siderophore-dependent iron acquisition by SirABC (58) or (iii) the export of bacteriocins (209).
Recently, a novel ABC transporter Pmt was identified in *S. aureus*. Pmt is encoded by the *pmtABCD* operon and was shown to be essential for the export of phenol-soluble modulins (PSMs) (40). PSMs, which include the δ-haemolysin, are peptide toxins and exhibit diverse functions in *S. aureus* pathogenesis (220).

Finally, there are the two export systems: **holins**, which are involved in the export of PG-degrading enzymes as proposed for the CidA and LrgA proteins (228) and natural cell **lysis**.

1.3 RND proteins

The resistance-nodulation-cell division (RND) family belongs to the multidrug resistance (MDR) efflux pumps. RND proteins are characterized by a typical structure of twelve TM domains with two extracytoplasmic loops between the TM1-2 and the TM7-8 revealing two repeats, which are thought to arise from internal gene duplication (Figure 7A) (239, 278). As an energy source they use the PMF. RNDs are found throughout all three domains of life (archeae, bacteria and eukaryotes) (278) and have been ascribed diverse functions, ranging from the export of virulence factors, including quorum sensing signals (223) to the morphogen receptor Ptc, which plays a role in *Drosophila melanogaster* development (137, 278). Two prominent representatives are; SecDF, an accessory component of the Sec secretion system and the well-studied acriflavine resistance protein B (AcrB) from *E. coli*.

AcrB together with the outer membrane protein TolC and the membrane fusion protein AcrA form the tripartite efflux system AcrAB-TolC (Figure 7B) (186). AcrA acts as a linker between AcrB and TolC as depicted in Figure 7C. The constitutively active RND pump is responsible for the efflux of a broad range of substrates, such as lipophilic antibiotics, dyes, detergents and host-derived substances, like bile salts (162, 193, 195, 196, 271). Substrate

specificity is determined by the periplasmic loops of AcrB (80) and it is assumed that the substrates are exclusively captured from the periplasm or the periplasm-inner membrane interface (193, 194). The driving energy for the drug pumping is from the proton motive force through the AcrB transmembrane domain (186, 250). Since bacterial multidrug efflux pumps reduce drug accumulation in the cell, they are often associated with antibiotic resistance (193).

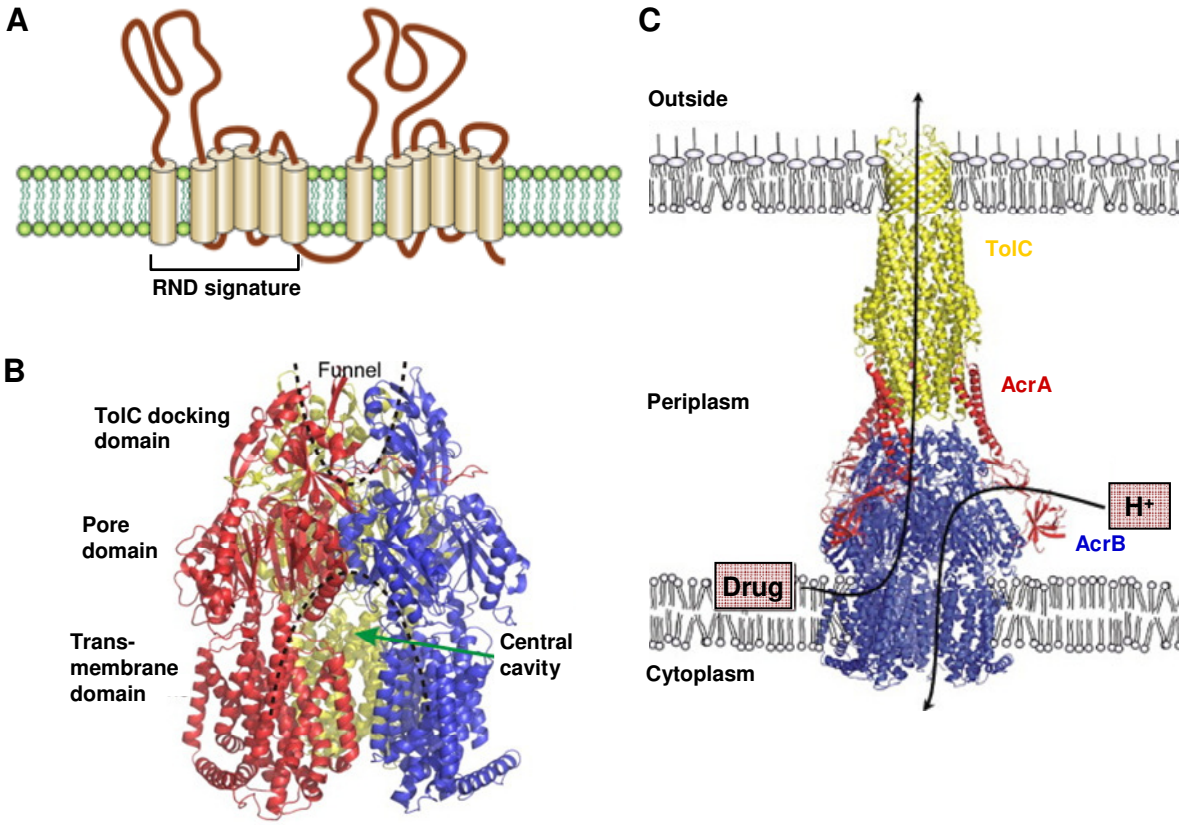

Figure 7. Models of RND proteins and the multidrug exporter AcrAB-TolC. (A) Topology model of a typical RND protein with twelve TM domains with two large hydrophilic loops. Taken from (127). (B) Side view of the trimeric RND protein AcrB of *E. coli* with its different domains. Taken from (249) (C) Schematic picture based on X-ray structures of AcrAB-TolC with the drug/proton flow. Modified from (249).

One eukaryotic AcrB homologue is the human RND protein Niemann-Pick C1 (NCP1). NCP1 can bind cholesterol and plays a crucial role in intracellular cholesterol trafficking, although its precise function still remains debated (60, 143, 278). The NCP1 disease leads to a lipid storage disorder, the accumulation of intracellular cholesterol and cell death. In 95 % of the cases, it is caused by mutations in the aa sequence of NCP1. NCP1 expressed in *E. coli* facilitated the import of acriflavine and oleic acid, but not cholesterol, revealing a transport function (60).

M. tuberculosis possesses several proteins described as mycobacterial membrane protein large (MmpL) belonging to the RND family. MmpL genes are often found in clusters involved in the biosynthesis of cell wall-associated glycolipids, which can make up to 60 % of the mycobacterial cell wall (268). Lipid transport functions have been described for MmpL7, MmpL8 and the essential protein MmpL3 (52, 130, 289).
So far, only one out of the 13 MmpL proteins was shown to play a role in antibiotic resistance. Over-expression of MmpL7 in *Mycobacterium smegmatis* leads to resistance towards isoniazid, a first-line antibiotic for treatment of tuberculosis (218, 232). Furthermore, MmpL7 is involved in phthiocerol dimycocerosate (PDIM) transport across the cell membrane (130). PDIM is a surface-exposed polyketide lipid and plays a major role in virulence by interacting with host cells (121, 130). Deletion of MmpL7 leads to impaired growth kinetics and lethality in a murine tuberculosis model (72).

So far, not much is known about RND proteins in low-G+C Gram-positive bacteria. *B. subtilis* has four RND proteins: YerP, YdfJ, YdgH and SecDF. YerP is involved in self-resistance to the cyclic lipopeptide surfactin and participates in the export of acriflavine and ethidium bromide (281). The second RND protein YdfJ is regulated by the TCS YdfHI. YdfJ contains a sterol sensing domain (252) and together with the membrane protein YdgH belongs to the MmpL family. The fourth RND protein is the auxiliary SecDF protein from the Sec secretion system. In contrast to *E.* coli, SecDF in *B. subtilis* is fused to one polypeptide. Deletion of *secDF* in *B. subtilis* leads to a cold-sensitive phenotype, which is aggravated when secretory proteins are over-expressed (24).

RND proteins in *S. aureus*

S. aureus has three uncharacterized proteins belonging to the RND family (Figure 8): (i) SecDF (SA1463) with 53 % aa identity compared to *B. subtilis* SecDF (ii) SA2056, a homologue of *B. subtilis* YerP with 46 % aa identity and (iii) SA2339, which has 46 % aa identity to YdfJ of *B. subtilis* and 31 % aa identity to MmpL7 of *M. tuberculosis*.

Similar as in *B. subtilis*, *S. aureus* **SecDF** is encoded by a monocistronic mRNA, but the genetic context of *secDF* resembles the one of *E. coli* with *yajC* lying downstream of *secDF* (Figure 8A). Two conserved charged residues Asp519 and Arg247 were shown to be crucial for SecDF activity in *E. coli* and point mutations of the counterparts in *T. thermophilus* Asp340Asn and Arg671Met were shown to abolish ion channel activity. These conserved aa

are also found in the *S. aureus* SecDF and correspond to Asp327 and Arg679. *recJ*, a predicted single-stranded DNA-specific 5'-3' exonuclease is situated upstream of *secDF*.

The second *S. aureus rnd* gene, ***sa2056***, lies downstream of the prominent non-ribosomal peptidyltransferase *femX* (Figure 8B), which catalyzes the addition of the first glycine to the pentaglycine bridge of the PG precursor. Previously, attempted knock-out experiments by colleagues failed (233), suggesting SA2056 to be important for viability. Knock-down experiments did not show any apparent phenotype and over-expression of SA2056 did not lead to a higher resistance against antibiotics (233). The gene lying downstream of *sa2056*, *sa2055* encodes a hypothetical protein with unknown function.

SA2339, the third *S. aureus* RND protein, is a member of the MmpL family. *sa2339* was shown to be highly induced in a transcriptional-profiling assay, when cells were treated with tea tree oil (55). Tea tree oil is known to exhibit broad-spectrum antimicrobial activity *in vitro* (35). Furthermore, microarray analysis showed *sa2339* to be positively affected directly or indirectly by the transcriptional regulator NorG (276). NorG was shown to bind to promoters of several multidrug efflux pumps encoding genes and to modulate resistance to quinolones and β-lactams (277). *sa2340*, which is situated upstream of *sa2339*, contains a helix-turn-helix (HTH) TetR-type DNA binding domain and is therefore categorized into to the transcriptional regulator TetR-family (Figure 8C). s*a2338* lying downstream of *sa2339* contains a FeoA domain (Fe^{2+} transport system protein A).

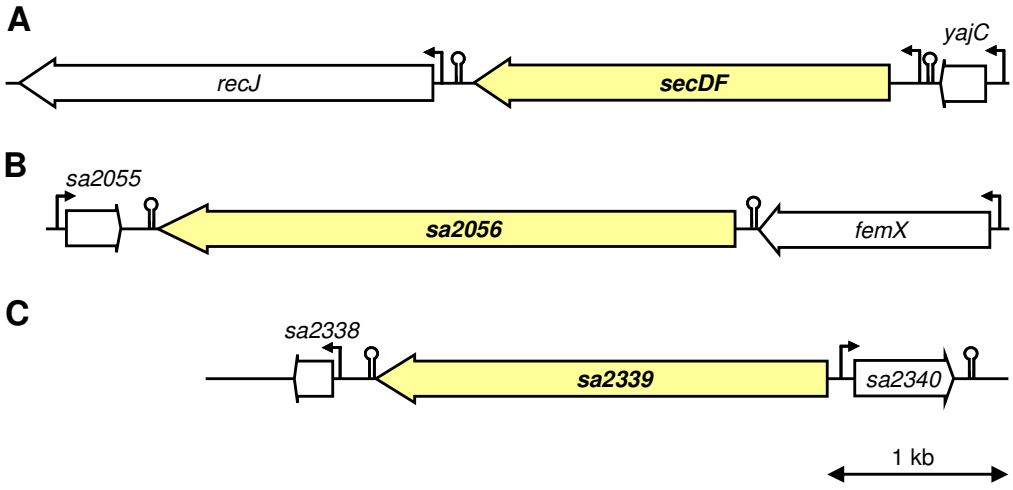

Figure 8. Genetic organization of *secDF*, *sa2056* and *sa2339* in *S. aureus* including predicted promoter and Rho-independent transcription terminators. (A) *secDF* locus (2.28 kb) (B) *sa2056* locus (3.168 kb) and (C) *sa2339* locus (2.49 kb) of *S. aureus* N315. Genes encoding proteins belonging to the RND family are coloured in yellow. Promoter (263) and terminator (264) predictions are depicted by arrows and hairpins, respectively.

2 Aims of this study

The RND proteins were first thought to be exclusive in Gram-negative bacteria (239). However, a phylogenetic analysis by Tseng *et al.* revealed RNDs to be ubiquitous and present in all three major kingdoms of life (278). So far, most studies of the RND proteins were performed in Gram-negative bacteria or *M. tuberculosis* and information on RNDs in low-G+C Gram-positive bacteria is scarce. Their diverse functions and in particular their contribution to antibiotic resistance by multidrug efflux transporters, make them an interesting target.

The aim of this study was to characterize the three RND proteins in the Gram-positive bacterium *S. aureus* and especially to elucidate their role in fitness, resistance and virulence.

This was achieved by the construction of three *S. aureus* RND single mutants using a new markerless deletion system. A thorough phenotype analysis was performed, consisting of growth and fitness studies under different stress conditions and a resistance profile analysis for several antibiotics, RND substrates and fatty acids.

Project I Analysis of the AcrB homologue SA2056

Due to the conserved genetic organization of *femX-sa2056* in *S. aureus* and the weak co-transcription of *sa2056* with *femX*, a role of SA2056 in cell wall biosynthesis seemed possible (233). In parallel to studies concerning the topology and identification of interaction partners, transcription and expression of SA2056 during growth were determined. To assess wether SA2056 plays a role in cell wall synthesis, the impact of *sa2056* deletion on the ultrastructure of the cell and autolysis was studied. Furthermore, the localization of SA2056-GFP recombinant proteins was analyzed to reveal its position during cell separation.

Project II Determination of SecDF in *S. aureus* resistance and expression of virulence factors

To elucidate the influence of SecDF on β-lactam resistance and the export of RND substrates in *S. aureus*, the *secDF* mutant was further analysed regarding the expression and transcription of several well known and Sec-dependent virulence factors. Cell morphology, autolytic activity and the expression of PBPs were studied.

Project III Defining the role of SecDF in *S. aureus* virulence

For examining the effect of protein secretion, a quantitative secretome analysis was performed. Follow up experiments comprised confirmation and complementation by Western blot and the verification in a second strain background. Consequences of the altered exoproteome for virulence of the *secDF* mutant was assessed by *in vitro* assays with human umbilical vein endothelial cells and an *in vivo* infection assay in a *Galleria mellonella* insect model.

3 Results

3.1 Project I

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Article

The *Staphylococcus aureus* Membrane Protein SA2056 Interacts with Peptidoglycan Synthesis Enzymes

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Abstract: The yet uncharacterized membrane protein SA2056 belongs to the ubiquitous RND (Resistance-Nodulation-cell Division) family of transmembrane efflux transporters. The *sa2056* gene is located downstream of *femX*, the gene encoding the essential, non-ribosomal peptidyl-transferase adding the first glycine in the staphylococcal cell wall pentaglycine interpeptide. Due to its proximity to and weak co-transcription with *femX*, we assumed that *sa2056* may somehow be involved in peptidoglycan synthesis. Specific antibodies against SA2056 showed that this protein is expressed during growth and present in the membrane fraction of cell preparations. Using a bacterial two hybrid system, SA2056 was shown to interact (i) with itself, (ii) with FemB, which adds glycines 4 and 5 to the peptidoglycan interpeptide and (iii) with the essential penicillin binding proteins,

PBP1 and PBP2, required for cell division and incorporation of the peptidoglycan into the cell wall. Unexpectedly, deletion of *sa2056* led to no phenotype regarding growth, antibiotic resistances or cell morphology; nor did *sa2056* deletion in combination with *femB* inactivation alter β-lactam and lysostaphin sensitivity and resistance, respectively, pointing to possible redundancy in the cell wall synthesis pathway. These results suggest an accessory role of SA2056 in *S. aureus* peptidoglycan synthesis, broadening the range of biological functions of RND proteins.

Keywords: *Staphylococcus aureus*; RND protein; FemABX; PBP; peptidoglycan; bacterial two-hybrid system

1. Introduction

One of the most common nosocomial human pathogens, *Staphylococcus aureus* can cause a variety of hospital- and community-acquired infections and intoxications. Treatment of this Gram-positive bacterium has become difficult due to its ability to rapidly develop resistance against virtually all currently used antibiotics. Genes potentially involved in cell wall synthesis, a pathway unique to bacteria, may represent novel targets for the therapy of staphylococcal infections.

The main component of the bacterial cell wall is a three-dimensional peptidoglycan meshwork whose backbone consists of the alternating saccharides N-acetylglucosamine and N-acetylmuramic acid (MurNAc). The characteristic pentapeptide branching off the MurNAc unit is synthesized in *S. aureus* by three non-ribosomal peptidyl-transferases; FemABX. Using Gly-tRNA as donor and the peptidoglycan precursor lipid II as substrate, they add in a sequential fashion five glycines to form a characteristic pentaglycine interpeptide (Gly_5) [1–4]. Cross-linking of adjacent peptidoglycan strands and anchoring of surface proteins, contributing to the virulence of *S. aureus*, occurs via this Gly_5-interpeptide [5]. An incomplete Gly_5 interpeptide leads to aberrant growth, requiring compensatory mutations to assure survival [6,7], while a complete lack is lethal [2]. Importantly, methicillin resistant *S. aureus* (MRSA) depend for high-level resistance on the correct formation of the peptidoglycan precursor, including a complete Gly_5 chain [8–10]. After transport across the cellular membrane, the peptidoglycan precursor is incorporated into the existing cell wall by the PBPs (for *p*enicillin *b*inding *p*rotein), exoplasmic enzymes catalyzing transglycosylation of the sugar moiety and transpeptidation of the Gly_5 chain. β-lactams, such as methicillin, inhibit the latter reaction by irreversibly binding to the active site of the transpeptidase domain.

The *orf* down-stream of *femX, sa2056*, encodes a putative 114.7 kDa protein with 12 predicted transmembrane domains belonging to the resistance-nodulation-cell division (RND) family. RND proteins are ubiquitous and have diverse biological functions, ranging from multidrug exporters, such as AcrB in *Escherichia coli* to morphogen receptors in *Drosophila melanogaster*, as found for patched (Ptc) (reviewed in [11]). SA2056 is annotated as a hydrophobic/amphiphilic exporter-1 (HAE1) family protein (subclass 2.A.6.2 in the Transporter Classification database; TCDB [12]), with 93% of the SA2056 amino acid sequence matching AcrB/AcrD/AcrF family motifs (Kyoto Encyclopedia of Genes and Genomes database; KEGG [13]).

The genetic organization *femX-sa2056* is conserved among all published annotated staphylococcal species. Previous attempts to knock-out *sa2056* had been unsuccessful, and Northern blot analyses had indicated co-transcription of *femX* and *sa2056* [14], suggesting *sa2056* to be essential and to have a cell wall-related function associated with *femX*.

Both *femX* and *sa2056* lie on the negative strand of the *S. aureus* chromosome and are separated by a 117 bp segment. Rho-independent transcription terminators are predicted by TransTermHP downstream of both *femX* and *sa2056* [15]. Apart from the promoter upstream of *femX*, the program softberry identified an additional putative promoter in the intergenic region between *femX* and *sa2056* [16]. Microarray analyses had shown *sa2056* to be slightly upregulated in response to daptomycin, peracetic acid and chlorination [17–19]. On the other hand, *sa2056* is downregulated by mupirocin and mitomycin and in a *graRS* and *clpP* mutant background [20–22]. These alterations are paralleled by *femX* only in the case of daptomycin or mupirocin challenge and in the *clpP* mutant, suggesting that transcription of *femX* and *sa2056* can occur simultaneously or autonomously, depending on the conditions. Interestingly, SA2056 was found to harbor single-nucleotide polymorphisms (SNPs) in an *in vitro* generated ceftobiprole-resistant *mecA*-negative COL variant [23]. In this strain, additional SNPs were present only in two other genes: in *pbp4* encoding the only low-molecular-weight PBP of *S. aureus*, PBP4 and in *gdpP*, influencing the levels of the second messenger c-di-AMP [24]. Both PBP4 and GdpP directly or indirectly play a role in cross-linking of peptidoglycan and β-lactam resistance [25–30], further supporting the hypothesis that also SA2056 could play a role in peptidoglycan synthesis.

In this study, analysis of the markerless *sa2056* knock-out mutant CQ33 [31] was extended to various growth and stress conditions. In addition, SA2056 was tested for interaction with peptidoglycan synthesis enzymes in a bacterial two hybrid system and in pull-down experiments. Although we could not find a phenotype for the mutant, we could show that SA2056 interacted with some of the FemABX factors and the PBPs, suggesting SA2056 to play a subsidiary role in peptidoglycan synthesis.

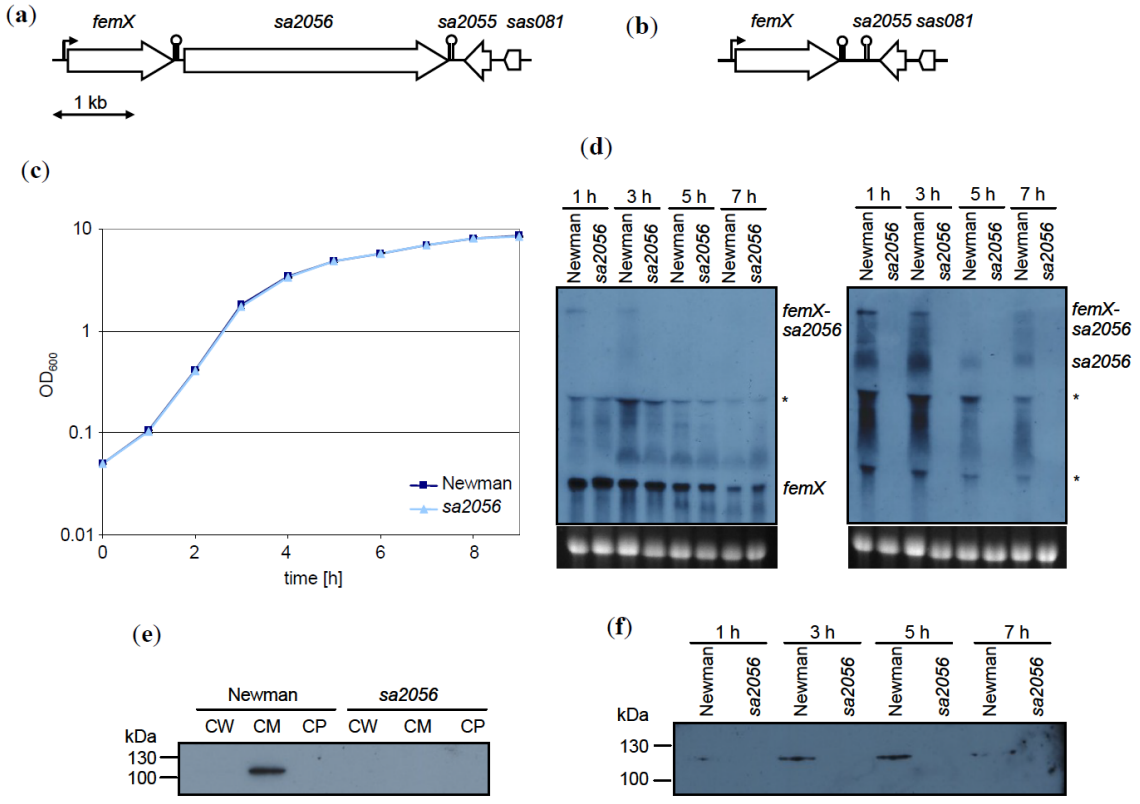
2. Results and Discussion

2.1. Expression of sa2056 During Growth

The transcriptional profile of *sa2056* was determined by Northern blot analyses with specific DIG-labeled probes against *femX* or *sa2056* (Figure 1). *sa2056* was transcribed mainly during exponential growth and partially co-transcribed with *femX*, as a 4.55 kb-transcript could be detected with both probes. Transcriptional start site determination by primer extension did not identify a promoter initiating a *sa2056*-specific mRNA (data not shown), suggesting that the mRNA of approximately 2.9 kb hybridizing only with the *sa2056* probe might result from processing of the 4.55 kb transcript. However, we cannot exclude the presence of an alternative promoter that could be active under different conditions than used here. The hairpin structure between *femX* and *sa2056* might function as a transcriptional or translational attenuator, further regulating SA2056 levels. Of interest, *femX* transcription (1.5 kb) was not altered by the deletion of *sa2056*.

Specific antibodies against a recombinant hexahistidine-tagged SA2056 protein were produced and used for Western blot analyses. SA2056 production was highest in late and post-exponential phase (Figure 1) and could be detected in the membrane part of fractionated wild-type cells and not in the *sa2056* mutant. Thus, *S. aureus* expressed SA2056 during growth, suggesting that it has a function in dividing cells.

Figure 1. Expression of *sa2056* in strain Newman and its *sa2056* mutant. Genetic organization of the *femX-sa2056* region in (**a**) the wild-type and (**b**) the *sa2056* mutant. Construction of the *sa2056* mutant is detailed in supplementary figure S1. (**c**) Growth curves from Luria-Bertani broth (LB) cultures monitored during 9 h. (**d**) Northern blot analyses of RNA samples taken after 1, 3, 5 and 7 h of growth. Digoxigenin (DIG)-labeled probes for *femX* (left panel) and *sa2056* (right panel) were used. Relevant bands are indicated. Ethidium bromide-stained 16S rRNA is shown as an indication of RNA loading. Bands that might be caused by interference of bulk 16S and 23S rRNA are designated by asterisks. Specific antibodies against SA2056 (114.7 kDa) were used for Western blot analyses of (**e**) cell wall (CW), cell membrane (CM) and cytoplasmic (CP) fractions isolated from exponentially growing cells and (**f**) membrane preparations from samples taken after 1, 3, 5 and 7 h of growth.



2.2. Analyses of Mutant Phenotype

Bacteria grew in LB during 7 h or 4 days without any apparent difference between wild-type and mutant concerning optical density or colony forming units as reported before [31]. Increasing or decreasing the temperature to 43 °C or 17 °C and addition of salt (1.5 M NaCl) or sucrose (1 M) to test osmotic stress conditions did not lead to any growth difference that might have indicated altered cell envelope stability. Biofilm formation was determined, but was found to be similar as in the wild-type. Both autolysis and ultrastructure of the cells, as determined by electron microscopy, were unchanged (data not shown).

Resistance levels were tested for different antibiotic classes, including β-lactams, glycopeptides or substances affecting peptidoglycan precursor synthesis and a variety of RND-substrates (Supplementary Table S1). Also, daptomycin was included, because this membrane-active antibiotic had been shown to induce *sa2056* transcription 2.04-fold [17]. However, MICs were virtually identical in wild-type and mutant. The *sa2056* mutant was found to be only moderately more resistant to hypochlorite compared to the parent (growth at 5 mM respectively 2.5 mM hypochlorite). No change in resistance was found for mitomycin, mupirocin, peracetic acid or puromycin (data not shown).

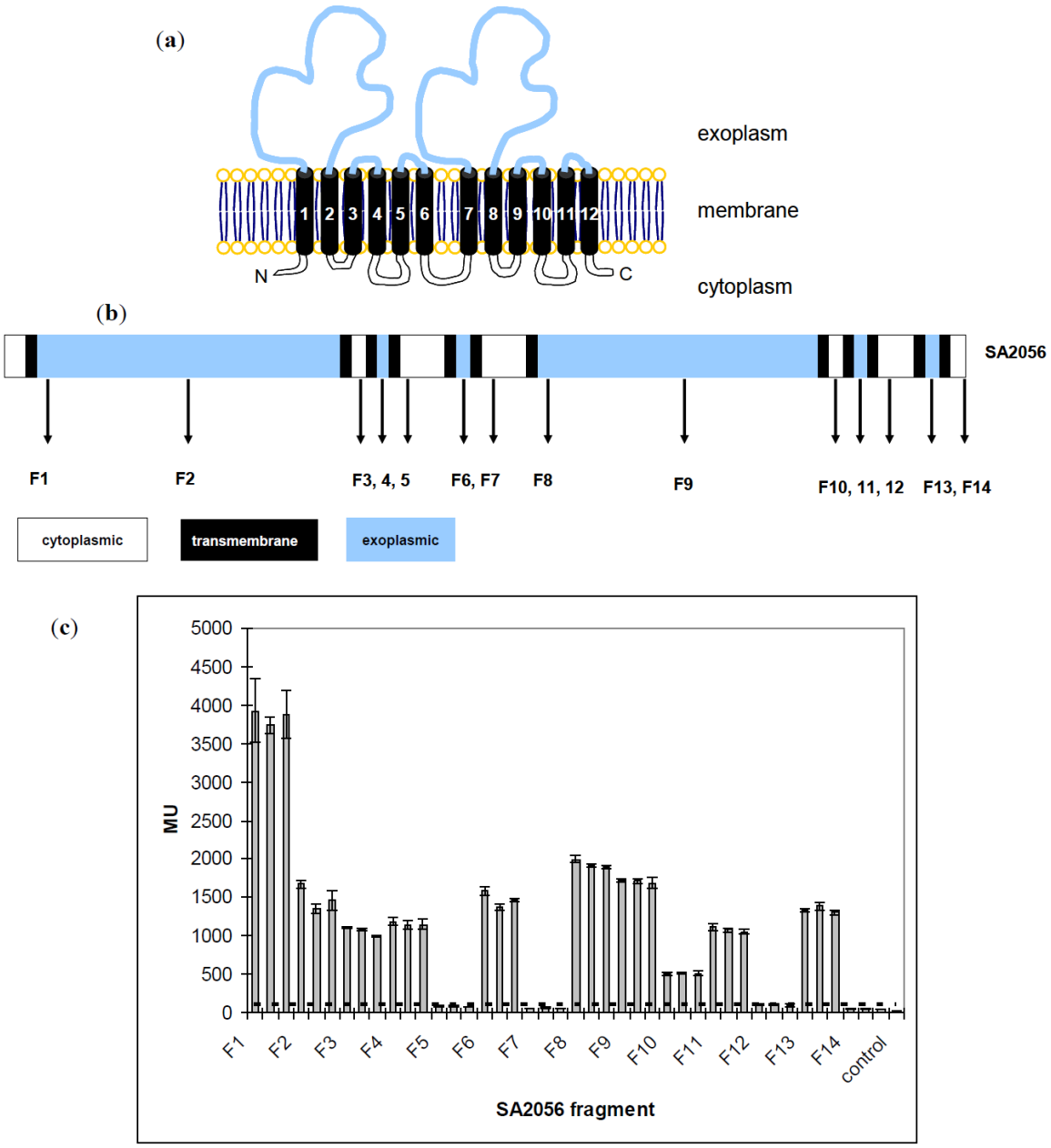
To see whether the presence of the methicillin resistance-mediating PBP, PBP2a, had any influence, we transformed Newman and Newman *sa2056* with the plasmid pME2, encoding *mecA* controlled by its promoter [32]. However, there was no difference in the expression of homogeneous resistance as deduced from population analysis profiles on oxacillin (data not shown).

These data suggested that, although expressed, *sa2056* is not required for *S. aureus* growth or stress tolerance under the conditions tested.

2.3. Topology of SA2056

The protein SA2056 is predicted to have 12 transmembrane (TM) domains and two large exoplasmic loops, displaying a typical RND topology with an internal symmetry [11]. Using the TMHMM program, putative TM regions were determined, and fragments containing increasing numbers of TMs (Supplementary Figure S2) were fused to the N-terminus of the *E. coli* alkaline phosphatase PhoA [33]. PhoA is widely used in topology studies, because it folds only in the exoplasm into an enzymatically active conformation [34–36]. Expression of the fusion proteins was confirmed by Western blot analyses (data not shown), and PhoA activity was determined (Figure 2). Topology was confirmed, except for fragments ending after TM2 and TM8. Sequence analyses with other membrane prediction programs (DAS, SOSUI, HMMTOP, MEMSAT) revealed ambiguities regarding the end of TM2 and the start of TM3, for which additional F3 constructs were made (F3a–e; S2) by adding up to five amino acids. However, all of the constructs directed PhoA to the exoplasm, suggesting that the short stretch between TM2 and TM3 might not allow PhoA to protrude into the cytoplasm. Similarly, the same might be true for TM8 and TM9, which are separated by 3–6 aa, depending on the program. Taken together, we could confirm the overall topology and show that SA2056 covers cytoplasmic, membrane and exoplasmic spaces. Thus, SA2056 has the potential to interact with proteins present in these different subcellular locations.

Figure 2. Analysis of SA2056 topology. (**a**) Model of SA2056 topology depicting cytoplasmic (white), membrane (black) and exoplasmic (blue) segments. The N- and C-terminus of the protein are both predicted to be located in the cytoplasm. (**b**) SA2056 fragments F1–14 cloned to the N-terminus of PhoA. (**c**) Activity of fusion proteins was measured in biological and technical triplicates; mean values for each clone are given, and the standard deviation is indicated. SA2056 fragments directing PhoA to the exoplasm were expected to produce values at least five times higher than the background levels (dashed line) measured in the *phoA*-negative *E. coli* strain CC118 (control).



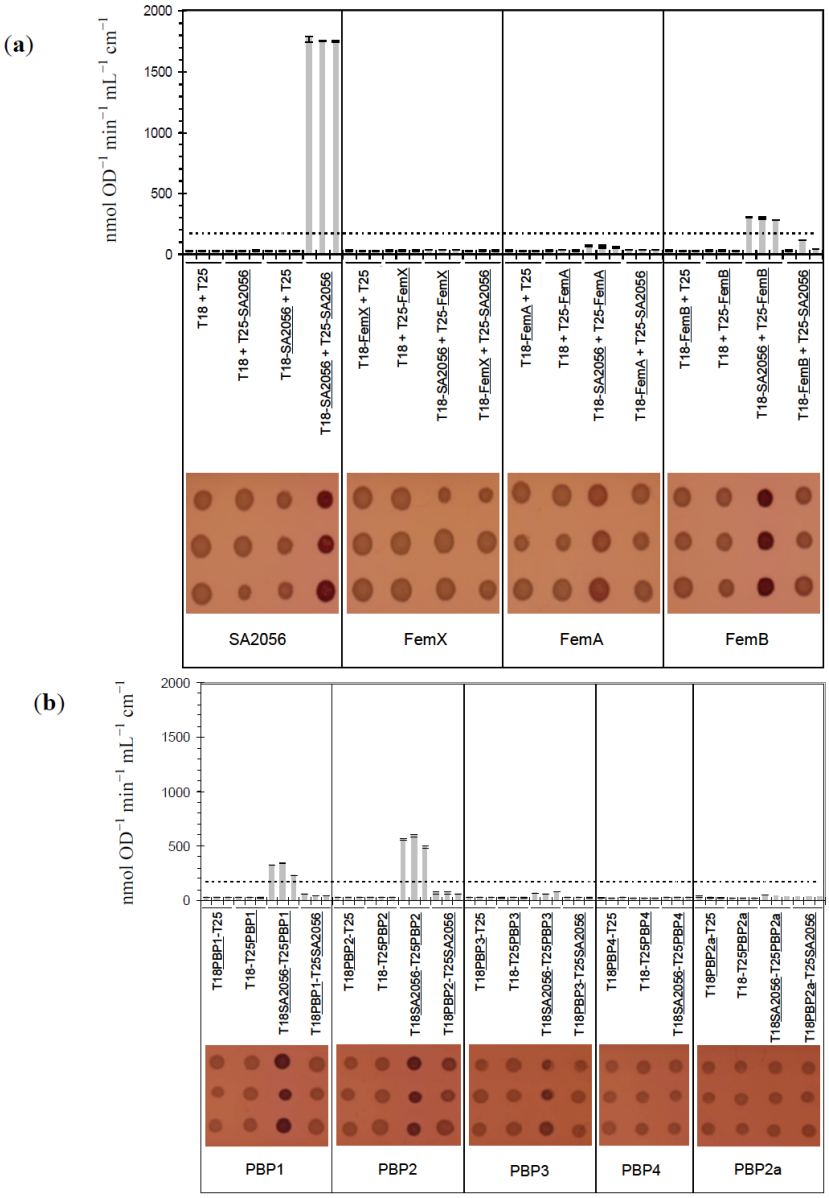
To monitor the localization of SA2056 in the cell, the green fluorescent protein (GFP) was fused to the C-terminus of SA2056, and expression of SA2056-GFP was visualized in exponentially growing cells (Supplementary Figure S3). Quantification of the fluorescence signal of the hemispherical and septal membrane showed that the signal from the septum was approximately twice as high, suggesting that the increased brightness was caused by the presence of two instead of one plasma membrane at the septum and not by a preferred localization of SA2056 to the septum. However, because of the relatively high background signal in the cytoplasm, no ultimate conclusion about the localization of SA2056 could be drawn. Discrete fluorescent patches, which could reflect a heterogeneous distribution of SA2056 in the membrane, were also observed in the membrane in dividing and non-dividing bacteria and were not influenced by the addition of methicillin (data not shown).

2.4. Interactions of SA2056 Identified in a Bacterial Two-Hybrid System

In parallel to the construction of a *sa2056* deletion mutant, interaction studies were performed using a bacterial two-hybrid system (BACTH) developed by Karimova *et al*. [37]. To test whether there was a physical link to peptidoglycan synthesis besides the transcriptional coupling to *femX*, interactions with the FemABX factors and the PBPs were examined. As the *E. coli* RND protein AcrB had been reported to form trimers [38], SA2056 was also tested for interaction with itself. Candidates were fused to the *Bordetella pertussis* adenylate cyclase CyaA domains T18 and T25, as described in Materials and Methods. pKT25 and pUT18-vectors encoding the fusion proteins were co-transformed into the *cya* negative *E. coli* reporter strain DHM1. Co-transformants were plated on MacConkey agar containing lactose as the only carbon source. Interacting partners bring the T18 and T25 domains close enough together to allow them to regain their catalytic function, *i.e.*, the conversion of ATP into cAMP. Production of cAMP was monitored on indicator plates, where the expression of cAMP-dependent enzymes, such as β-galactosidase, leads to the degradation of lactose, acidification of the medium and color formation. To estimate the strength of interaction, β-galactosidase activity was determined. As negative controls, vectors encoding only T18 or T25 were combined with candidate proteins.

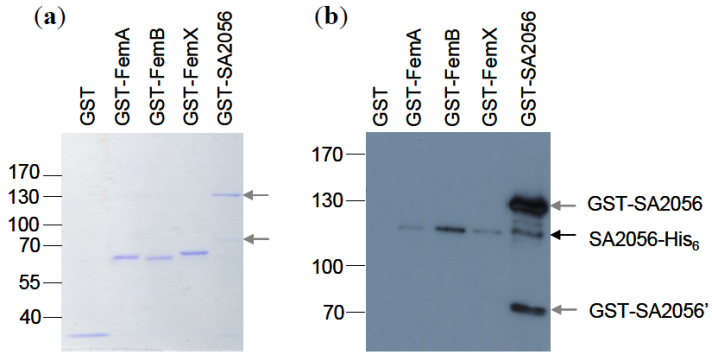
For pUT18-*pbp4*/pKT25-*sa2056* co-transformations, no viable clones were obtained; the same plasmids were used successfully in other transformations, suggesting that this particular combination was unfavorable for the cells and that the plasmids themselves were not toxic. The interaction of PBP4 with SA2056 was therefore tested only in one orientation. For every combination, three representative clones were analyzed (Figure 3). The highest value of all negative controls was multiplied by five to set the threshold for significant interactions. SA2056 was found to strongly interact with itself, suggesting that, although not extruding RND substrates, this protein has the potential to form homotrimers like other RND proteins. Possibly due to an unfavorable conformation of the T25-SA2056 protein, SA2056 was able to interact with FemB, PBP1 and PBP2 only when fused to the T18 domain.

Figure 3. SA2056 interactions determined using the bacterial two-hybrid system. (**a**) SA2056 interactions with itself and the FemABX factors. (**b**) SA2056 interactions with penicillin binding proteins (PBPs). Three representative co-transformants containing the plasmids indicated were analyzed regarding β-galactosidase activity, which was determined by measuring the formation of o-nitrophenol (top). Means of three technical replicates and their standard deviation are shown. The threshold corresponding to the highest negative control value multiplied by five is indicated by a dashed line. Alternatively, the ability of co-transformants to degrade lactose to lactate was tested on MacConkey agar (bottom), where acidification of the medium leads to pink colonies.



To exclude that endogenous *E. coli* proteins were mediating or hindering interactions, pull-down experiments with purified recombinant proteins were performed. Proteins were tagged with glutathion-S-transferase (GST) or a hexahistidine (His_6) peptide. SA2056-GST or GST alone was bound to glutathion-sepharose, blocked with BSA and incubated with His_6-tagged interaction candidates. After washing, bound proteins were detached with sample buffer and separated on denaturing polyacrylamide gels (Figure 4). Similar amounts of GST-tagged bait proteins were present in the reactions, as determined by Coomassie staining (Figure 4a). SA2056 was confirmed to interact with itself and FemB and was found to very weakly interact also with FemA and FemX under these conditions (Figure 4b). Pull-down experiments with recombinant PBP-His_6 proteins were unsuccessful, possibly requiring further optimization of assay conditions allowing PBPs to interact with SA2056. For instance, co-factors might be required that are only present in the cell or a membrane environment.

Figure 4. Pull-down experiments. Recombinant glutathion-S-transferase (GST)-tagged SA2056 and FemABX proteins were incubated with SA2056-His_6 and aliquots of bound proteins were separated on denaturing polyacrylamide gels for Coomassie staining (**a**) or (**b**) transferred to a polyvinylidene fluoride (PVDF)-membrane for Western blot analysis using specific antibodies against SA2056. Relevant bands are indicated. GST (26 kDa), GST-FemA (74.1 kDa), GST-FemB (74 kDa), GST-FemX (74.3 kDa), SA2056-His_6 (115 kDa). Probably due to degradation, an additional band (~70 kDa; GST-SA2056') was visible in the preparation of GST-SA2056 (147.2 kDa) and detected with the antibodies. For appropriate separation, a 10%-(a) and a 7.5%-polyacrylamide gel (b) were used. GST-tagged proteins had a tendency to run slightly faster than expected from their predicted mass.



2.5. Resistance Phenotype of a femB sa2056 Double Mutant

Since FemB was found in both *E. coli* and *in vitro* experiments to interact with SA2056, a *femB sa2056* double mutant was constructed to determine whether the lack of *sa2056* in the compromised *femB* single mutant leads to a phenotype. A *femB* transposon mutation was transduced into the Newman *sa2056* mutant, and the resulting *femB sa2056* double mutant was tested for any alterations in β-lactam or lysostaphin resistance compared to the *femB* mutant, which is known to have a reduced β-lactam and an increased lysostaphin resistance [39,40]. However, the *femB sa2056* mutant showed similar resistance to cefoxitin and lysostaphin, as did the single mutant *femB* (data not shown), suggesting that under the conditions tested *sa2056* does not play a major role in *S. aureus,* and thus, a

major function in the last steps of peptidoglycan precursor synthesis is unlikely. This was also supported by testing of recombinant SA2056 in the previously described *in vitro* peptidoglycan synthesis assay, where no alterations in pentaglycine interpeptide synthesis was found upon addition of SA2056 (data not shown) [4].

In *S. aureus*, two additional RND proteins are present: SecDF and SA2339, a homologue of MmpL that may be involved in lipid transport. While the importance of SecDF for *S. aureus* resistance and expression of virulence factors has been recently described, the function of SA2339 has not yet been identified. Like *sa2056*, deletion of *sa2339* leads to no phenotype regarding growth or resistance [31]. It is possible that SA2056 and SA2339 share functional redundancy, similar to the *S. aureus* LytR-CpsA-Psr proteins [41] and that deletion of just one of the genes does not impair *S. aureus* sufficiently to produce a phenotype. Alternatively, SA2056 might be part of a complex network involving several players, where the absence of just one factor has little impact on *S. aureus* under the conditions tested here.

3. Experimental

3.1. Bacterial Strains and Growth Conditions

Strains and plasmids used in this study are listed in Supplementary Table S2. Bacteria were grown aerobically at 37 °C in Luria-Bertani broth (LB), where nothing else is mentioned. Good aeration for liquid cultures was assured by vigorously shaking flasks with an air-to-liquid ratio of at least 4. For growth curves, strains were grown in triplicate, and means with standard deviations were determined.

Antibiotic susceptibilities were determined using Etest strips (AB-Biodisk, Solna, Sweden), containing exponential gradients of active components, on MH agar plates with an inoculum of a 0.5 McFarland standard, corresponding to 10^8 cells/mL. Minimal inhibitory concentrations (MICs) were read after 24 h of incubation. Alternatively, broth microdilution methodology was used. For qualitative susceptibility determination, bacterial 0.5 McFarland suspensions were swabbed across agar plates containing appropriate concentration gradients of test substances.

To sample RNA and protein, cells from overnight cultures were used to inoculate prewarmed LB to an optical density at 600 nm (OD_{600}) of 0.05, corresponding to 10^7 cells/mL.

3.2. Construction of MRSA Strains

The plasmid pME2 containing the *mecA* promoter and gene from strain COLn [32] was introduced into strains of interest, as described in [31].

3.3. Northern Blot Analyses

Total RNA was isolated as described previously [42] by using a FastRNA kit and a Fastprep reciprocating shaker (Bio 101). For Northern blots, 5 to 10 µg of total RNA per lane was separated on a 1.5% agarose-20 mM guanidine thiocyanate gel and transferred overnight onto a positively charged nylon membrane (Roche, Rotkreuz, Switzerland). The blots were hybridized with specific digoxigenin-labeled DNA probes, which were produced using a PCR DIG probe synthesis kit (Roche). Primers used are listed in Supplementary Table S3. Data shown were confirmed in at least two independent experiments.

3.4. Expression of Recombinant Proteins

Genes encoding FemABX, SA2056, PBP1-4 and 2a were amplified from genomic DNA using primers listed in supplementary table T3. Amplicons were cloned into pET24b(+) using NheI/XhoI or BamHI/XhoI. In the case of pGEX-2T, PCR products were inserted into BamHI/EcoRI digested plasmids.

His_6-tagged and GST-tagged FemABX factors were purified as described in [43]. For the membrane proteins SA2056 and the PBPs, the *E. coli* BL21 derivative CE43 was used, since it had been selected for increased membrane protein production [44]. Transformants were grown in LB at 37 °C. At an OD_{600} of 1.5, the expression of the recombinant proteins was induced with 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG). Bacteria were then grown for 20 h at 25 °C and were collected by centrifugation.

For purification of His_6-tagged proteins, pellets were resuspended in 50 mM Tris-HCl pH 7.5, 500 mM NaCl, 20 mM imidazol and 0.5% N-lauroylsarcosine. Protease inhibitors (Complete EDTA-free, Roche) were added as recommended by the manufacturer. Cells were lysed on ice for 30 min with 2 mg/mL lysozyme, 0.15 mg/mL DNase and 0.075 mg/mL RNase. The cleared lysate was gently mixed for 2 h at 4 °C with Ni-NTA beads (Qiagen, Hombrechtikon, Switzerland). Beads were collected and washed with 50 mM Tris/HCl pH 7.5, 500 mM NaCl, 20 mM imidazol, followed by a second wash with the same buffer containing 50 mM imidazol. Proteins were eluted with 50 mM Tris/HCl pH 7.5, 500 mM NaCl, 1 mM *n*-dodecyl-β-D-maltoside (DDM), 100 mM imidazol followed by a second round of elution with the same buffer containing 200 mM imidazol. Proteins were stored at 4 °C or, supplied with 20% glycerol, at −20 °C.

GST-tagged proteins were isolated similarly with minor changes: Resuspension was done in 50 mM Na_2HPO_4 pH 7.8, 150 mM NaCl, 10 mM DDM. Glutathion (GSH)-Sepharose 4B (GE Healthcare) was used for binding of the GST-tagged proteins, which were washed once with the same buffer and then eluted with 10 mM GSH, 50 mM Tris-HCl pH 8. Ten mM DDM was added only in the case of the membrane proteins SA2056, PBP1-4 and 2a.

3.5. S. aureus Cell Fractionation

Based on the method used by Schneewind *et al.* [45], cell wall, cell membrane and cytoplasmic fractions of bacteria grown until an OD_{600} of 1 were prepared, as described in [31]. Representative data from two independent experiments are shown.

3.6. Western Blot Analyses

Recombinant His-tagged SA2056 was prepared as described above and used for the production of specific rabbit antibodies (Davids Biotechnology, Regensburg, Germany). For Western blots, 10 µg protein was loaded and separated by SDS-10% polyacrylamide gel electrophoresis. Page Ruler (Thermo Scientific, Waltham, MA, USA) was used as a molecular size marker. Gels were either stained with Coomassie or transferred onto nitrocellulose (Hybond; Amersham Biosciences, Glattbrugg, Switzerland) or polyvinylidene fluoride (PVDF; Immobilon-P, Millipore, Zug, Switzerland) membranes. Membranes were blocked with skim milk and preincubated with 40 µg/mL human immunoglobulin G (Calbiochem, Darmstadt, Germany) to saturate any immunoglobulin-

binding proteins and, thereby, prevent cross-reactivity of antigen-purified rabbit antibodies against SA2056. Horseradish peroxidase-conjugated goat anti-rabbit secondary antibody (Jackson ImmunoResearch Laboratories, Inc., Suffolk, UK) was diluted 1:10,000 and detected with SuperSignal West Pico solutions (Thermo Scientific).

3.7. Construction of phoA *Fusions and PhoA Activity Assay*

The gene fragments of interest were cloned into the 5' XhoI and 3' KpnI sites of plasmid pHA-1, which carries a *phoA* gene lacking both the 5' segment coding for the signal sequence and the first five residues of the mature protein [36]. Between the SA2056-fragment and the PhoA moiety, an 18 amino acid linker was present. The constructs were transformed into the *phoA*-negative *E. coli* strain CC118, which is not able to use arabinose and galactose. For each construct, three transformants were used for the determination of PhoA activity.

Three mL LB medium was inoculated with 30 µL overnight culture and grown at 37 °C to an OD_{600} of 0.5. The protein expression was induced with 0.2% arabinose during 1 hour at 37 °C. One mL of the culture was centrifuged at maximal speed in a microcentrifuge, washed with 1 mL ice cold 1 M Tris-HCl (pH 8.0) and resuspended in 1 mL ice cold 1 M Tris-HCl (pH 8.0). After measuring the OD_{600}, 3 aliquots (0.05, 0.1, 0.2 mL) were adjusted to a total volume of 0.5 mL with ice cold 1 M Tris-HCl (pH 8.0). Cells were permeabilized by adding 20 µL chloroform and 10 µL 0.01% SDS and incubation at 37 °C for 5 min. Addition of 0.5 mL of 2 mM p-nitrophenyl phosphate (pNPP, Sigma-Aldrich, St. Louis, MO, USA) in 1 M Tris-HCl (pH 8.0) initiated the reaction. After 10 min incubation at 37 °C, the reaction was stopped by adding 0.2 mL 0.5 M K_2HPO_4 (pH 8.0). The cell debris was removed by centrifugation, and the absorption was measured at 550 and 420 nm to determine residual cell debris, respectively, and color formation by pNPP degradation. Miller Units were calculated using the following formula:

$$MU = 1000 \times (OD_{405} - OD_{550}) / (OD_{600} \times \text{suspension volume assayed} \times \text{time})$$

3.8. Topology Prediction Programs

Five topology prediction programs were used: TMHMM [46], DAS [47], HMMTOP [48], MEMSAT [49], SOSUI [50].

3.9. Bacterial Two-Hybrid System

Candidate genes were amplified from genomic DNA using primers listed in Supplementary Table S3 and cloned into pUT18 and pKT25 vectors [37] using the restriction sites PstI and KpnI. Fifty µL of RbCl-competent DHM1 cells were co-transformed with 20 ng of each plasmid and plated on Difco MacConkey agar containing lactose (BD, No. 212123), 25 µg/mL kanamycin (Km) and 100 µg/mL ampicillin (Ap). Transformants were grown at 30 °C, and three representative clones from a minimum of 50 clones were restreaked for further experiments. One mL LB with 0.5 mM IPTG, 25 µg/mL Km, 100 µg/mL Ap was inoculated with one clone and grown at 30 °C for 16 h. Cells were centrifuged and stored at −20 °C until use. For spotting, 0.5 mL LB was inoculated with 5 µL overnight culture, of which 0.1 mL were transferred into microtiter dishes and used for spotting on MacConkey agar plates.

For determination of β-galactosidase activity, frozen pellets were resuspended in 1 mL Z buffer (60 mM Na_2HPO_4, 40 mM NaH_2PO_4, 10 mM KCl, 1 mM $MgSO_4$ pH 7) and OD_{600} was determined. 1 mL Z buffer was used as control and treated like the samples. For each clone, measurements were made in triplicate. One hundred µL aliquots were added to 900 µL Z buffer, 35 µL chloroform, and 35 µL 0.1% SDS was added. Cells were vortexed during 10 s for permeabilization, followed by an incubation step at 28 °C for 5 min. 0.2 mL of a 0.4% *o*-nitrophenol-β-galactopyranoside (ONPG) solution in buffer Z was added and incubated for 5 min at 28 °C. The reaction was stopped by adding 0.5 mL of a 1 M Na_2CO_3 solution. Cell debris was removed by centrifugation during 1 min at full speed. The supernatant was used to measure the absorption of the β-galactosidase product *o*-nitrophenol at 420 nm and to determine light scattering of any remaining particle at 550 nm. Activity, one unit corresponding to the hydrolyzation of 1 nmol of ONPG per min at 28 °C, was calculated using the following formula and is given in nmol OD^{-1} min^{-1} cm^{-1}:

$$activity = \frac{OD_{420} - (1.75 \times OD_{550})}{OD_{600} \times time \times vol} - \frac{blank \times 1\ nmol \times 1.7\ mL}{0.0045\ mL \times 1\ cm}$$

3.10. Pull-Down Experiments

Per reaction, 2 µg recombinant GST-tagged protein was bound to 10 µL of GSH-sepharose 4B slurry by mixing 1 h at 4 °C in binding buffer (50 mM Na_2HPO_4 pH 7.8, 150 mM NaCl, 1 mM DDM). Sepharose was washed four times with binding buffer and blocked with 5 mg/mL bovine serum albumin (BSA) in binding buffer for 0.5 h at 4 °C. Sepharose was washed four times with interaction buffer (40 mM Tris-HCl pH 7.5, 10 mM $MgCl_2$, 1 mM DDM, 0.2% BSA). Two µg recombinant His_6-tagged protein was added, and the reactions were mixed for 1 h at room temperature. Sepharose was washed four times with wash buffer (40 mM Tris-HCl pH 7.5, 10 mM $MgCl_2$, 1 mM DDM, 50 mM NaCl). Bound material was detached by adding 20 µL of sample buffer (4.4 M urea, 2.7% non-idet P-40, 2.7% β-mercaptoethanol, 0.16 M Tris-HCl pH 6.8, 6.2% SDS, 4.5% glycerol, bromophenol blue) and heated for 5 min at 65 °C. 7 µL were separated on a denaturing 7.5 or 10% polyacrylamide gel. 10%-gels were stained with Coomassie to visualize GST-tagged proteins and to exclude that any other *E. coli* proteins were present that might have mediated interactions. 7.5%-gels were blotted onto a PVDF-membrane and used for Western blots with specific rabbit anti-SA2056 or rabbit anti-His_6 (Abcam) antibodies, which were detected as described above. Representative data from two independent experiments are shown.

4. Conclusions

Under standard laboratory conditions, the yet uncharacterized RND protein, SA2056, is expressed in *S. aureus* and, thus, must be assumed to have a function. SA2056 can interact with itself, suggesting that it could form a trimer and work as an efflux pump; the substrate is likely to be very specific, as deletion of *sa2056* had no influence on resistance against typical RND substrates or a range of antibiotics. The interaction found between SA2056 and FemB using two different methods hints at a possible, subsidiary role in peptidoglycan synthesis or cell division, but could also be of a more general nature in coordinating processes occurring at the membrane. This yet uncharacterized role could represent a novel aspect of the functional diversity of RND proteins. In the case of *S. aureus*, the

function of SA2056 might be redundant with a second uncharacterized RND protein, SA2339, or be of importance only under certain conditions that were not tested here and remain to be identified.

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Conflict of Interest

The authors declare no conflict of interest.

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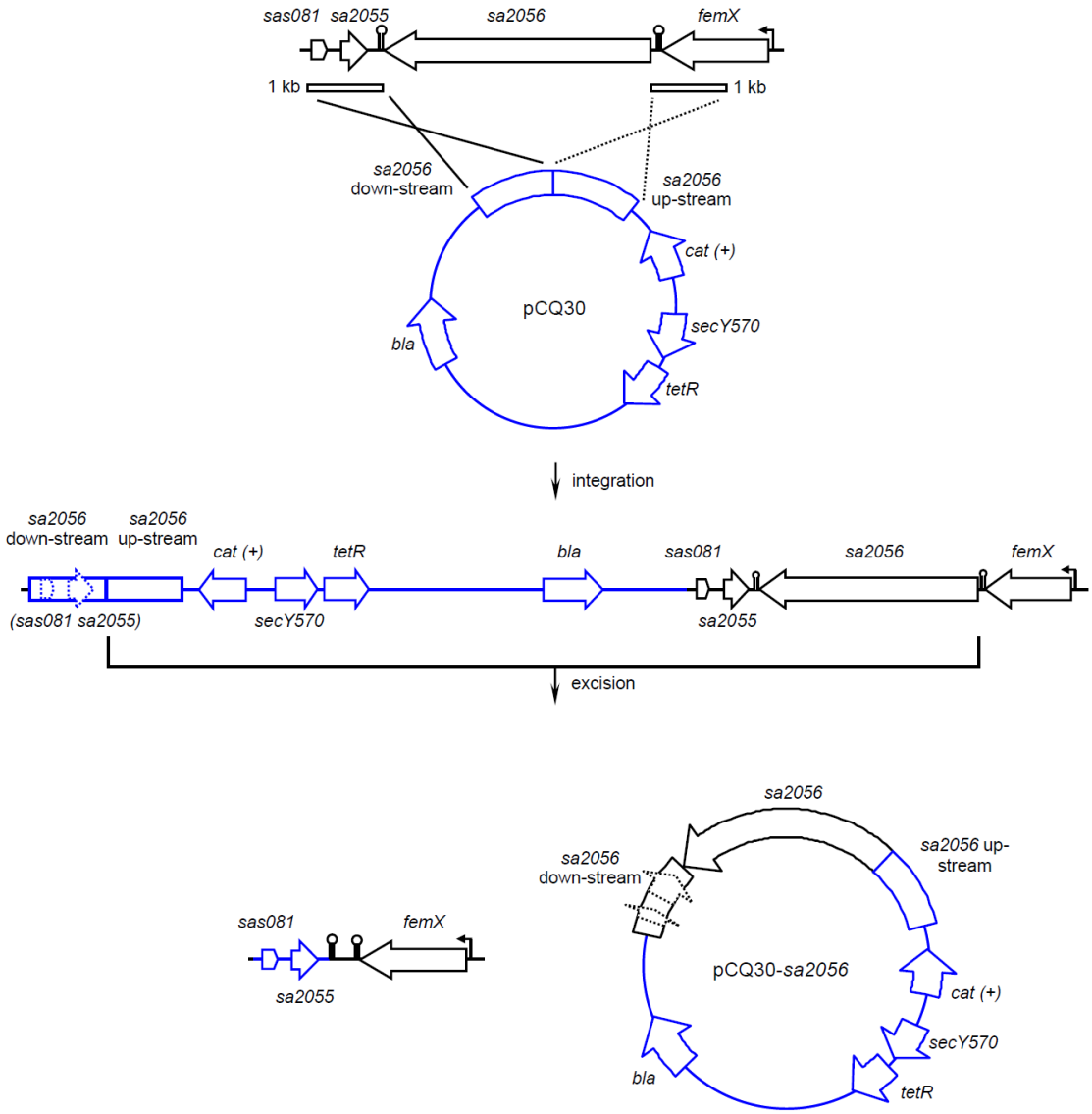
Author contribution

Design of study: BBB, HGS, MMS; construction of strains: ALK, CQ, EH, MMS; construction of plasmids: ALK, CQ, EH, MGP, SR, TS; transcription and transcriptional start site analysis: CQ; Western blot: CQ, MMS; analysis of mutant phenotypes: CQ, MMS; topology: ALK, EH, MMS; localization studies: CQ, DA, IW, MGP, TS; bacterial two-hybrid system: EH, MMS; pull-down experiments: MMS; prepared manuscript: MMS; reviewed manuscript: All.

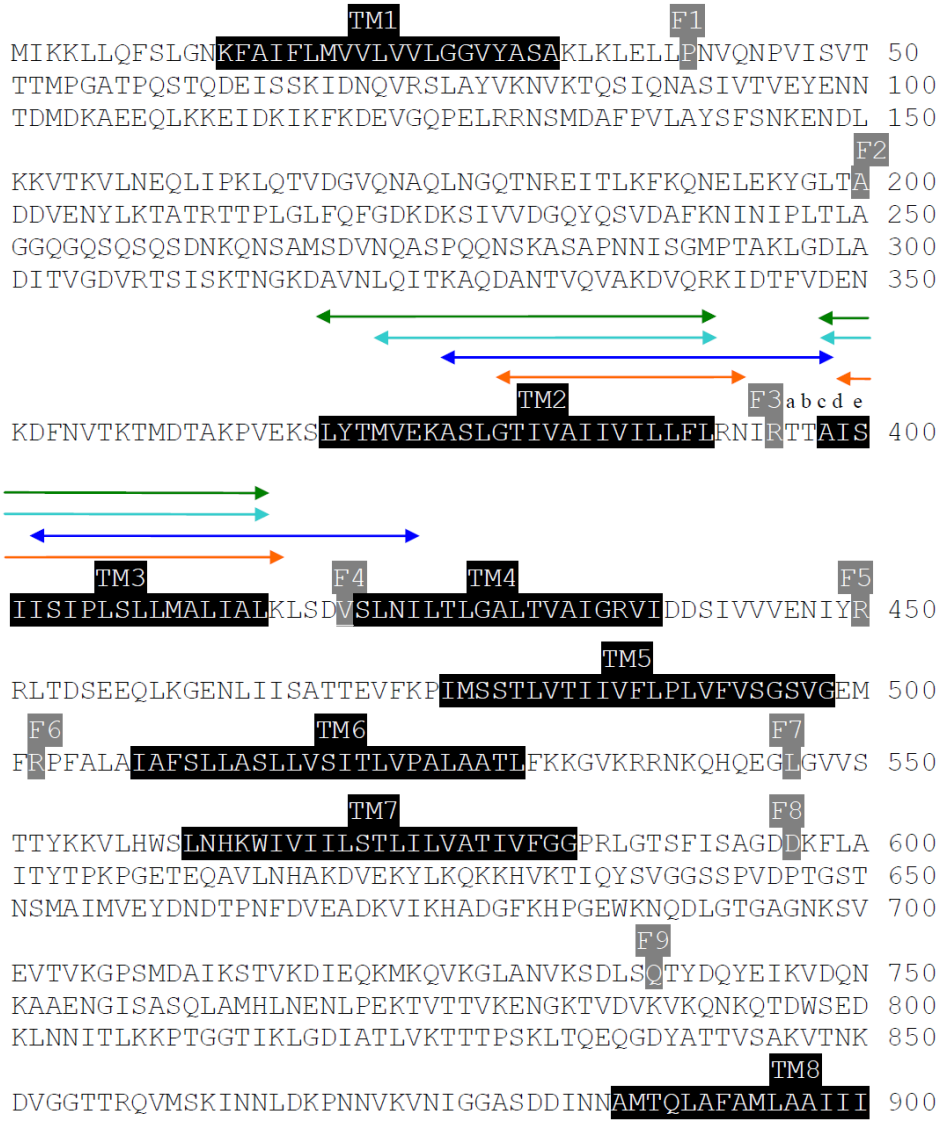
Supplementary Material

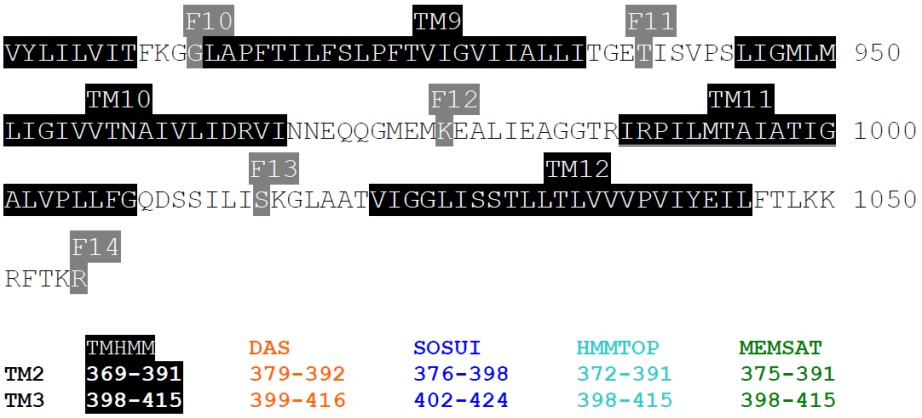
Supplementary Figure S1. Construction of the *sa2056* mutant [1]. *sa2056* was excised by a three-step procedure developed by Bae *et al.* [2]: First, the temperature-sensitive plasmid pCQ30 was integrated at 43 °C either up- or down-stream of *sa2056* by homologous recombination. Only the resulting chromosomal organization of the recombination symbolized on the left is given. Next, the plasmid was allowed to excise together with the *sa2056* gene at permissive temperature (30 °C). Finally, bacteria were selected for plasmid loss.



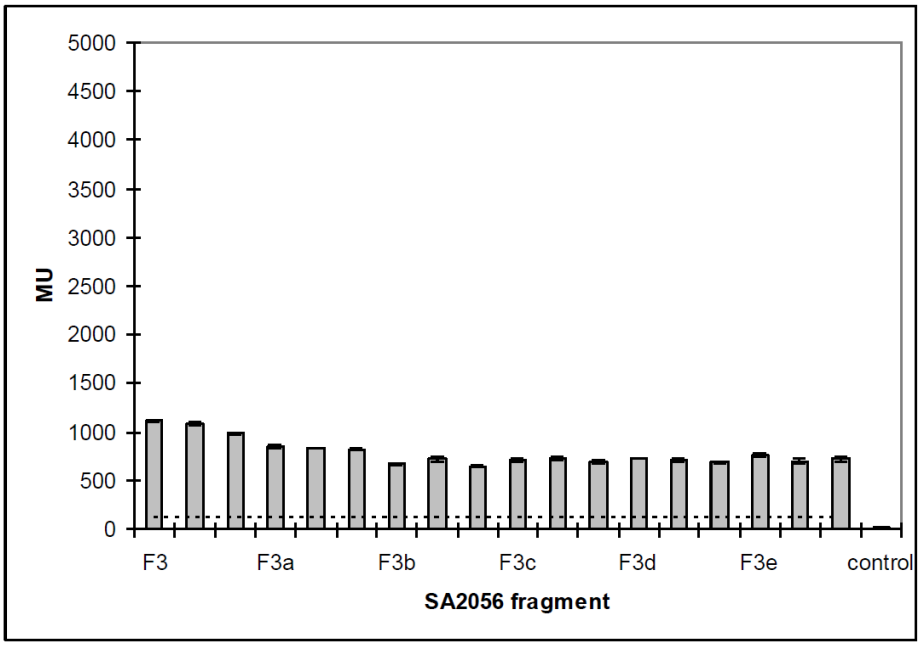
Supplementary Figure S2. (**a**) Amino acid sequence of SA2056. Transmembrane (TM) regions predicted by THMMH and C-termini of fragments (F) fused to PhoA are indicated. For TM2 and TM3, predictions of additional programs are depicted. Extra amino acids added to F3 are indicated (F3a–e). (**b**) Activity of fusion proteins was measured in biological and technical triplicates; mean values for each clone are given and the standard deviation is indicated. SA2056 fragments directing PhoA to the exoplasm were expected to produce values at least five times higher than the background levels (dashed line) measured in the *phoA*-negative *E. coli* strain CC118 (control).

(a)

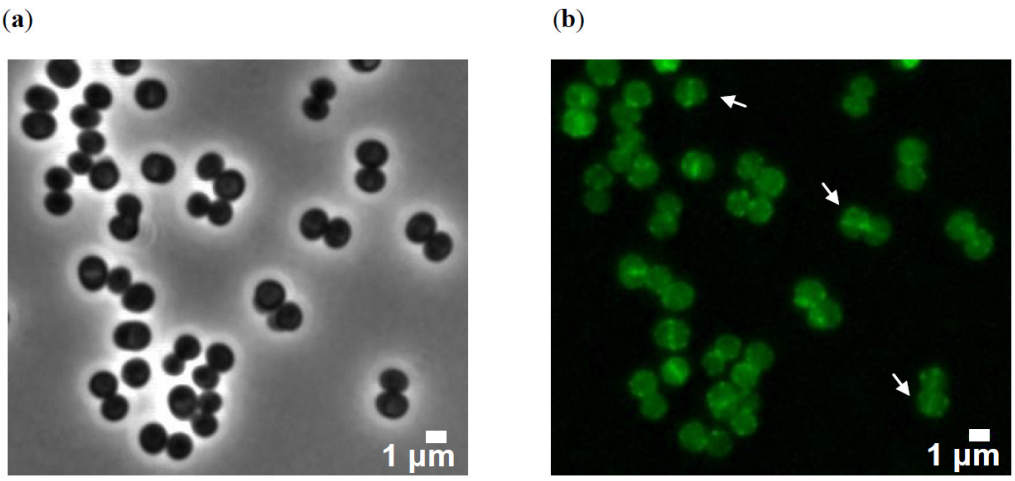



F10
TM9
F11
VYLILVITFKGGLAPFTILFSLPFTVIGVIIALLITGETISVPSLIGMLM 950
TM10
F12
TM11
LIGIVVTNAIVLIDRVINNEQQGMEMKEALIEAGGTRIRPILMTAIATIG 1000
F13
TM12
ALVPLLFGQDSSILISKGLAATVIGGLISSTLLTLVVVPVIYEILFTLKK 1050
F14
RFTKR
TMHMM
DAS
SOSUI
HMMTOP
MEMSAT
TM2 369-391 379-392 376-398 372-391 375-391
TM3 398-415 399-416 402-424 398-415 398-415

(b)


5000
4500
4000
3500
3000
2500
2000
1500
1000
500
0
MU
F3
F3a
F3b
F3c
F3d
F3e
control
SA2056 fragment

Supplementary Figure S3. Localisation of SA2056. Exponentially grown *S. aureus* expressing SA2056-GFP under the control of the *sa2056* promoter was visualised by (**a**) phase contrast or (**b**) fluorescence microscopy as described below. Arrows indicate examples of dividing bacteria with visible septa and SA2056-patches. Bars indicate the size of 1 µm.



The 3'-region of SA2056 ($SA2056_{666nt}$) was amplified from genomic DNA using primers listed in supplementary table T2. The $SA2056_{666nt}$ fragment was cloned to the 5' end of *gfpmut1* in pSG5082 using the XhoI and HindIII restriction sites, yielding pCQ44 [3]. Following the transformation of pCQ44 into *E. coli* DH5α (CQ44), the suicide vector was integrated into *S. aureus* RN4220 (CQ48). To confirm correct integration, a PCR with subsequent sequencing of the region was performed.

CQ48 was grown in tryptic soy broth (TSB, Difco) until exponential phase, washed once in PBS (8 g NaCl, 0.2 g KCl, 2.68 g Na_2HPO_4-$7H_2O$, 0.24 g KH_2PO_4, pH 7.4) and resuspended therein. A drop of bacterial suspension was spotted on a microscope slide overlaid with a thin layer of 1 % agarose in PBS and covered with a cover slip. Cells were visualised using a Zeiss Axio Observer.Z1 microscope and the Metamorph v. 7.5 software (Molecular Devices). Pictures were acquired with the Photometrics CoolSNAP HQ2 camera (Roper Scientific), which was connected to the microscope. Pictures were analysed with the ImageJ software [4].

Supplementary Table S1. Resistance profiles of strains Newman and *sa2056*.

		MIC [µg/mL]	
	Substance	**Newman**	***sa2056***
Cell wall synthesis inhibitors	Cefoxitin	6	6
	Oxacillin	0.38	0.38
	Teicoplanin	4	6
	Vancomycin	5	6
	Lysostaphin	0.125–0.25	0.125–0.25
	D-cylcoserine	8	8
	Fosfomycin	0.25	0.25
	Ramoplanin	1	1
	Nisin	4	4
	Mersacidin	32	32
	Bacitracin	8	8
RND substrates	Acriflavine	8	8
	EtBr	1–2	1–2
	SDS	64	64
Others	Daptomycin	2	2
	Clindamycin	0.94	0.94
	Chloramphenicol	4	3
	Tetracycline	0.19	0.25
	Gentamicin	0.75	1
	Erythromycin	0.25	0.25
	Novobiocin	0.0313	0.0313
Fatty acids	Capric acid	512	512
	Linoleic acid	16	16
	Cis-6-hexadecenoic acid	64	64

Supplementary Table S2. Strains and plasmids used in this study.

Strains	Relevant genotype and phenotype	Reference or source
S. aureus		
Newman	Clinical isolate (ATCC 25904), *rsbU*$^+$	[5]
RN4220	NCTC 8325-4 r$^-$ m$^+$	[6]
CQ33	Newman Δ*sa2056*	[1]
CQ38	Newman Δ*sa2056* pME2, Tc^r, Mc^r	[1]
CQ39	Newman pME2, Tc^r, Mc^r	[1]
CQ48	RN4220 *sa2056*::pCQ44, SA2056-GFP, Em^r	This study
MS146	Newman *femB*::Tn551, Em^r, Lss^r	This study
MS147	Newman Δ*sa2056 femB*::Tn551, Em^r, Lss^r	This study
UT34-2	NCTC 8325 *mec* Ω2006(*femB*::Tn551), Em^r, Lss^r	[7]
E. coli		
BL21	Expression strain, DE3 (*E. coli* B F$^-$ *ompT hsdS*$_B$ *gal dcm*),λ prophage carrying T7 polymerase	Novagen
CE43	Membrane protein overproducer selected from BL21	[8]
CC118	Reporter strain for PhoA fusion, Δ(*ara-leu*)7697 Δ*lacX74* Δ*phoA20 galE galK*	[9]
CQ44	DH5α pCQ44, Ap^r	This study
DH5α	Cloning strain (F− Φ80*lac*ZΔM15 Δ(*lac*ZYA-*arg*F)U169 *rec*A1 *end*A1 *hsd*R17 (rk−, mk+) *pho*A *sup*E44 *thi*-1 *gyr*A96 *rel*A1 λ−]	Invitrogen
DHM1	BACTH reporter strain, *cya*	[10]
Plasmids		
pCQ44	Suicide vector, SA2056$_{666nt}$-GFP fusion at C-terminus, Ap^r, Em^r	This study
pET24b(+)	Expression vector, N-terminal T7-Tag or C-terminal His$_6$-Tag, T7 promoter, Km^r	Novagen
pET24b(+)-*femA*	Expression vector, *femA* with His$_6$-Tag at the C-terminus, Km^r	[11]
pET24b(+)-*femB*	Expression vector, *femB* with His$_6$-Tag at the C-terminus, Km^r	[11]
pET24b(+)-*femX*	Expression vector, *femX* with His$_6$-Tag at the C-terminus, Km^r	This study
pET24b(+)-*sa2056*	Expression vector, *sa2056* with His$_6$-Tag at the C-terminus, Km^r	This study
pGEX-2T	Expression vector, N-terminal GST-Tag, Ap^r	GE Healthcare
pGEX-2T-*femA*	Expression vector, *femA* with GST-Tag at the N-terminus, Ap^r	This study
pGEX-2T-*femB*	Expression vector, *femB* with GST-Tag at the N-terminus, Ap^r	This study
pGEX-2T-*femX*	Expression vector, *femX* with GST-Tag at the N-terminus, Ap^r	This study
pGEX-2T-*sa2056*	Expression vector, *sa2056* with GST-Tag at the N-terminus, Ap^r	This study
pHA-1(*yedZ*)	PhoA fusion expression plasmid containing *yedZ* (XhoI-KpnI) with *phoA* fused to the 3'-end, *araB* promoter	[12]
pHA-F1-F14	PhoA fusion vectors, *sa2056* fragments encoding F1-F14 fused to the 5'-end of *phoA*	This study
pKT25	BACTH vector, MCS at the C-terminus of the CyaA domain T25, Km^r	[10]
pKNT	BACTH vector, MCS at the N-terminus of the CyaA domain T25, Km^r	[13]
pKT25-*femA*	BACTH vector, *femA* fused to the C-terminus of T25, Km^r	[11]
pKT25-*femB*	BACTH vector, *femB* fused to the C-terminus of T25, Km^r	[11]

Table S2. *Cont.*

Strains	Relevant genotype and phenotype	Reference or source
pKT25-*femX*	BACTH vector, *femX* fused to the C-terminus of T25, Km^r	This study
pKT25-*pbp1*	BACTH vector, *pbp1* fused to the C-terminus of T25, Km^r	[14]
pKT25-*pbp2*	BACTH vector, *pbp2* fused to the C-terminus of T25, Km^r	[14]
pKT25-*pbp3*	BACTH vector, *pbp3* fused to the C-terminus of T25, Km^r	This study
pKNT25-*pbp4*	BACTH vector, *pbp4* fused to the N-terminus of T25, Km^r	[14]
pKT25-*pbp2a*	BACTH vector, *pbp2a* fused to the C-terminus of T25, Km^r	This study
pKT25-*sa2056*	BACTH vector, *sa2056* fused to the C-terminus of T25, Km^r	This study
pSG5082	Suicide vector, for c-terminal GFP fusion, Ap^r, Em^r	[3]
pUT18	BACTH vector, MCS at the N-terminus of the CyaA domain T18, Ap^r	[10]
pUT18C	BACTH vector, MCS at the C-terminus of the CyaA domain T18, Ap^r	[10]
pUT18C-*femA*	BACTH vector, *femA* fused to the C-terminus of T18, Ap^r	[11]
pUT18C-*femB*	BACTH vector, *femB* fused to the C-terminus of T18, Ap^r	This study
pUT18C-*femX*	BACTH vector, *femX* fused to the C-terminus of T18, Ap^r	This study
pUT18C-*pbp1*	BACTH vector, *pbp1* fused to the C-terminus of T18, Ap^r	[14]
pUT18C-*pbp2*	BACTH vector, *pbp2* fused to the C-terminus of T18, Ap^r	This study
pUT18C-*pbp3*	BACTH vector, *pbp3* fused to the C-terminus of T18, Ap^r	[14]
pUT18-*pbp4*	BACTH vector, *pbp4* fused to the N-terminus of T18, Ap^r	[14]
pUT18C-*pbp2a*	BACTH vector, *pbp2a* fused to the C-terminus of T18, Ap^r	This study
pUT18C-*sa2056*	BACTH vector, *sa2056* fused to the C-terminus of T18, Ap^r	This study

MCS, multiple cloning site; Ap^r, ampicillin resistant; Cm^r, chloramphenicol resistant; Em^r, erythromycin resistant; Lss^r, lysostaphin resistant; Mc^r, methicillin resistant; Tc^r, tetracycline resistant.

Supplementary Table S3. Primers used in this study.

Primer	Sequence 5'-3'	Use	Reference
CQ10	TCACCCTCTCCACTGACAGA	Confirmation pCQ44 integration	This study
CQ31	AGTGTGGGGAACATACTTAAGTG	Confirmation pCQ44 integration	[3]
CQ33	ATCGAAGCAGGCGGTACTA	Sequencing CQ48	This study
CQ72	TATAAGCTTTCGTTTAGTGAATCGTTT	Construction of pCQ44	This study
CQ73	AAACTCGAGCAAGAACAAGGAGATTATGC	Construction of pCQ44	This study
CQ74	CATCACTTGTCGTGTTGTGC	Sequencing CQ48	This study
EH4	CTGGTACCTTAGTTGAATATACCTGTTAATCCAC	Construction of pUT18C-*pbp2*	This study
EH34	CCGCTCGAGATGATAAAAAAGCTATTAC	Construction of pHA1-F1-14	This study
EH35	CCGGGTACCGGTAGTAATTCTAATTTCA	Construction of pHA1-F1	This study
EH36	ATAGGTACCGCAGTCAACCCATATTTTTC	Construction of pHA1-F2	This study
EH37	ACGGGTACCCGAATGTTTCTTAAAAACAG	Construction of pHA1-F3	This study
EH38	GATGGTACCACATCACTCAATTTCAGAG	Construction of pHA1-F4	This study
EH39	CCGGGTACCCGATAAATATTTTCAACAA	Construction of pHA1-F5	This study
EH40	TATGGTACCCTAAACATTTCGCCTACTG	Construction of pHA1-F6	This study
EH41	ATCGGTACCAATCCTTCTTGATGTTGT	Construction of pHA1-F7	This study
EH42	ATTGGTACCTTATCGTCACCTGCTG	Construction of pHA1-F8	This study
EH43	TATGGTACCTGCGATAAATCAGATTTGAC	Construction of pHA1-F9	This study
EH44	GCGGGTACCCCACCTTTAAATGTAATA	Construction of pHA1-F10	This study
EH45	TAGGGTACCGTTTCTCCTGTGATTAATAG	Construction of pHA1-F11	This study
EH46	TATGGTACCTTCATCTCCATGCCC	Construction of pHA1-F12	This study
EH47	GTGGGTACCGAAATAAGAATCGAGCTAT	Construction of pHA1-F13	This study
EH48	CATGGTACCCGTTTAGTGAATCGTT	Construction of pHA1-F14	This study
EH50	AACTGCAGGACGGAAAACAAAGGATCTTC	Construction of pUT18C-*pbp2*	This study
MS79	ATGGGATCCTGCGAAGCAAAAAATTAAAATTAA	Construction of pET24b-*pbp1*	This study
MS80	TTACTCGAGGTCCGACTTATCCTTG	Construction of pET24b-*pbp1*	This study
MS81	TTGGGATCCCTTAAAAAGACTAAAAGAAAAATCAAATG	Construction of pET24b-*pbp3*	This study
MS82	TTACTCGAGTTTGTCTTTGTCTTTATTTTTATC	Construction of pET24b-*pbp3*	This study
MS83	ATGGGATCCCAAAAATTTAATATCTATTATCATCATTT	Construction of pET24b-*pbp4*	This study
MS84	TTACTCGAGTTTTCTTTTTCTAAATAAACGATTG	Construction of pET24b-*pbp4*	This study
MS85	ATGGGATCCCAAAAAGATAAAAATTGTTCCACT	Construction of pET24b-*mecA*	This study
MS86	TTACTCGAGTTCATCTATATCGTATTTTTTATT	Construction of pET24b-*mecA*	This study
MS106	CTAGAATTCTTTTCGTTTTAATTTACGAGATATTT	Construction of pGEX-2T-*sa2056*	This study
MS107	CGTGGATCCATAAAAAAGCTATTACAATTTTC	Construction of pGEX-2T-*sa2056*	This study
MS108	CTAGAATTCCTATCGTTTAGTGAATCGT	Construction of pGEX-2T-*sa2056*	This study
MS109	GTAAGATCTGAAAAGATGCATATCACTAATC	Construction of pGEX-2T-*femX*	This study
MS116	GCAGGTACCCTATTTCTTTAATTTTTTACG	Construction of pKT25-*femB* and pUT18C-*femB*	This study
MS117	GTTGAATTCCTATTTCTTTAATTTTTTACG	Construction of pGEX-2T-*femB*	This study
MS118	CTAGAATTCCTATTTTCGTTTTAATTTACGAG	Construction of pGEX-2T-*femX*	This study
MS155	ATTGGTACCGTACGAATGTTTCTTAAAAACAG	Construction of pHA1-F3a	This study
MS156	GAAGGTACCGTCGTACGAATGTTTCTTAAAAAC	Construction of pHA1-F3b	This study
MS157	ATAGGTACCGCCGTCGTACGAATGTTTC	Construction of pHA1-F3c	This study
MS158	GATGGTACCCCAATTGCCGTCGTACGAATGTTTC	Construction of pHA1-F3d	This study

Table S3. *Cont.*

Primer	Sequence 5'-3'	Use	Reference
MS159	GATGGTACCGAAATTGCCGTCGTACGAATG	Construction of pHA1-F3e	This study
SR2	CGAGCTAGCGAAAAGATGCATATCACTAATC	Construction of pET24b-*femX*	[15]
SR3	GCACTCGAGTTTTCGTTTTAATTTACG	Construction of pET24b-*femX*	[15]
SR71	CGTCTCGAGTCGTTTAGTGAATCGTTTTTT	Construction of pET24b-*sa2056*	This study
SR73	GCAGCTAGCATAAAAAAGCTATTACAATTTTCTTT	Construction of pET24b-*sa2056*	This study
SR100	GCACTGCAGGAAATTTACAGAGTTAACTG	Construction of pUT18C-*femB*	[11]
SR101	GCACTGCAGTGAAATTTACAGAGTTAACTG	Construction of pKT25-*femB*	[11]
SR103	CTACTGCAGGGAAAAGATGCATATCAC	Construction of pKT25-*femX*	[11]
SR104	CATCTGCAGTGGAAAAGATGCATATCAC	Construction of pUT18C-*femX*	[11]
SR105	GCAGGTACCTATTTTCGTTTTAATTTACG	Construction of pKT25-*femX*	[11]
SR106	GTTGGATCCAAGTTTACAAATTTAACAGCTA	Construction of pGEX-2T-*femA*	[11]
SR107	GTTGAATTCCTAAAAAATTCTGTCTTTAACTTT	Construction of pGEX-2T-*femA*	[11]
SR108	CAAGGATCCAAATTTACAGAGTTAACTGTTAC	Construction of pGEX-2T-*femB*	[11]

Restriction sites are underlined.

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3.2 Project II

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RESEARCH ARTICLE Open Access

Contribution of SecDF to *Staphylococcus aureus* resistance and expression of virulence factors

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Abstract

Background: SecDF is an accessory factor of the conserved Sec protein translocation machinery and belongs to the resistance-nodulation-cell division (RND) family of multidrug exporters. SecDF has been shown in *Escherichia coli* and *Bacillus subtilis* to be involved in the export of proteins. RND proteins can mediate resistance against various substances and might be of relevance in antimicrobial therapy. The role of RND proteins in *Staphylococcus aureus* has not yet been determined.

Results: Markerless deletion mutants were constructed to analyze the impact of the so far uncharacterized RND proteins in *S. aureus*. While the lack of Sa2056 and Sa2339 caused no phenotype regarding growth and resistance, the *secDF* mutant resulted in a pleiotropic phenotype. The *secDF* mutant was cold sensitive, but grew normally in rich medium at 37°C. Resistance to beta-lactams, glycopeptides and the RND substrates acriflavine, ethidium bromide and sodium dodecyl sulfate was reduced. The *secDF* mutant showed an aberrant cell separation and increased spontaneous and Triton X-100 induced autolysis, although the amounts of penicillin-binding proteins in the membrane were unchanged. The impact of *secDF* deletion on transcription and expression of specific virulence determinants varied: While coagulase transcription and activity were reduced, the opposite was observed for the autolysin Atl. A reduction of the transcription of the cell wall anchored protein A (*spa*) was also found. The accumulation of SpA in the membrane and lowered amounts in the cell wall pointed to an impaired translocation.

Conclusions: The combination of different effects of *secDF* deletion on transcription, regulation and translocation lead to impaired cell division, reduced resistance and altered expression of virulence determinants suggesting SecDF to be of major relevance in *S. aureus*. Thus SecDF could be a potential target for the control and eradication of *S. aureus* in the future.

Background

Staphylococcus aureus is a frequent colonizer of the human body as well as a serious human pathogen. It is known for its adaptability to diverse environments. It can cope with stress factors and acquire resistances to antibiotics thus rendering treatment difficult. *S. aureus* can cause a wide range of infections, mainly due to an impressive arsenal of virulence determinants comprising cell surface components and excreted factors interacting with the host system. Transport of proteins to the cell surface and secretion to the extracellular space is mediated through different transport systems [1] of which the general protein secretion system Sec plays a prominent role in protein export and membrane insertion.

Sec-mediated translocation has best been studied in *Escherichia coli* and is catalyzed by the essential SecYEG protein complex (reviewed in [2]). The motor ATPase SecA or a translating ribosome is believed to promote protein export by driving the substrate in an unfolded conformation through the SecYEG channel. The accessory SecDF-YajC complex facilitates protein export and membrane protein insertion efficiency in vivo [3], possibly via the control of SecA cycling [4]. The large exoplasmic loops of the integral membrane proteins SecD and SecF have been shown to be required for increasing protein translocation by a yet unknown mode of action [5]. While *secDF* disruption leads to a cold-sensitive phenotype and defects in protein translocation [6], the absence of YajC, which interacts with SecDF, causes

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only a weak phenotype [7]. SecYEG has been shown to interact with the SecDF-YajC complex [8]. YidC, a protein that is proposed to mediate membrane integration and the assembly of multimeric complexes, can also interact with SecDF-YajC to take over SecYEG-dependent membrane proteins [9].

Data on the *S. aureus* Sec system is scarce: SecA and SecY have been shown to be important, respectively essential, for growth by using antisense RNA [10]. Deletion of *secG* resulted in an altered composition of the extracellular proteome, which was aggravated in a *secG secY2* double mutant [11]. Deletion of *secY2* alone, which together with *secA2* belongs to the accessory Sec system [12], did not show any effect on protein translocation. As in the Gram-positive bacterium *Bacillus subtilis*, in *S. aureus* the accessory SecD and SecF proteins are fused to form a single protein (Sa1463), which was identified in membrane vesicles [13]. However, the chromosomal organization in *S. aureus* resembles the one of *E. coli*, with *yajC* lying immediately upstream of *secDF*. Furthermore, SecDF was identified in a surface-exposed peptide epitope screen by using a cell shaving technique [14] and expression was found to be slightly higher in a COL *sigB* deletion mutant [15]. SecDF is postulated to be essential in *S. aureus* according to a mutagenic screen [16].

SecDF belongs to the resistance-nodulation-cell division (RND) family of multidrug export pumps, that is conserved and widely distributed in all three major kingdoms of life [17]. RND proteins have a wide substrate specificity and diverse functions ranging from the efflux of noxious host derived substances, such as bile salts by *E. coli* [18] to the involvement of eukaryotic efflux pumps in cholesterol homeostasis in humans [19]. Multiple antibiotic resistance can be associated with these exporters, as they often recognize a broad range of substrates, thereby diminishing drug accumulation in the cell [20,21]. *S. aureus* possesses two additional uncharacterized RND proteins, namely Sa2056, located downstream of the essential *femX* [22], and Sa2339 (MmpL homologue).

Results

Construction of the *rnd* mutants

To evaluate the role and impact of the RND proteins in *S. aureus*, markerless deletion mutants were constructed in the sequenced and well-characterized clinical strain Newman. SecDF, Sa2056 and Sa2339 were found to be dispensable, as we obtained null mutants by allelic replacement of the corresponding genes using the pKOR1 system of Bae et al. [23]. The mutants were confirmed to have generally retained genome stability and to carry the desired modification in the corresponding locus as described in methods.

Deletion of *sa2056* and *sa2339* had no apparent effect on *S. aureus* when evaluating growth and resistance properties (data not shown), suggesting that they may be important under other conditions than applied in this study. The following report is therefore focused on the *secDF* mutant and its phenotype.

Transcription of *secDF* and growth phenotype of the *secDF* mutant

Transcription of *secDF* was monitored from early exponential to early stationary phase and found to result mainly in a monocistronic mRNA. *secDF* was strongest transcribed during early growth phase and declined towards stationary phase (Figure 1A). As expected, no transcripts were detected in the *secDF* deletion mutant. Transcriptional profiles were restored in the mutant by introducing the complementing plasmid pCQ27, containing the *secDF* gene from Newman with its endogenous promoter (data not shown).

At 37°C no significant difference was observed when comparing the growth curves of the wild type strain Newman and the mutant (Figure 1B). However, colonies of *secDF* mutants were smaller on blood agar compared to the wild type (83% ± 5.1 of the wild type's cross section). TEM pictures were prepared from exponentially growing cells. In contrast to the wild type (Figure 2A) and the complemented mutant (Figure 2C), displaying normally shaped cells with a maximum of one septum, the *secDF* mutant had difficulties in separating daughter cells (Figure 2B and 2D). This resulted in clusters with sometimes multiple and wrongly placed septa. At least 400 cells per strain were analyzed, showing that 20.4 ± 8.7% of the mutant cells could not divide correctly whereas this was only the case in 0.3 ± 0.7% for the wild type and 0.9 ± 1.3% for the complemented mutant.

As *secDF* knock out mutants in *B. subtilis* and *E. coli* show a cold sensitive phenotype [6,24], growth of the *S. aureus secDF* mutant was tested at 15°C. The temperature drop affected the *secDF* mutant approximately after two generations, causing a notably reduced growth rate with a subsequent halt in growth after 24 h. The plasmid pCQ27, but not the empty vector pCN34, significantly restored growth at 15°C (Figure 1B).

Increased susceptibility of the *secDF* mutant towards RND-substrates, β-lactam and glycopeptide antibiotics

Since multidrug resistance can be mediated unspecifically by RND exporters [21,25], we characterized the resistance profile of the *secDF* mutant by testing several different classes of antibiotics and typical RND-substrates [26,27]. The *secDF* mutant showed increased susceptibility to the RND substrates acriflavine, ethidium bromide and sodium dodecyl sulfate (SDS) on gradient plates (Figure 3). Furthermore, a distinct increased

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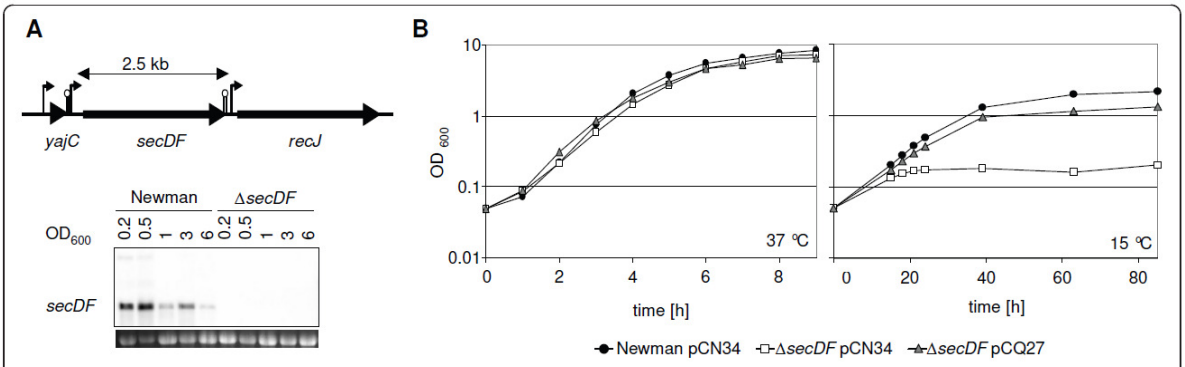

Figure 1 Growth characteristics of the *secDF* mutant. (**A**) Genetic context of *secDF* in *S. aureus* and Northern blot analysis of *secDF* transcription during growth. Predicted promoter and terminators are depicted. Ethidium bromide-stained 16S rRNA is shown as an indication of RNA loading. **(B)** Growth of Newman and the *secDF* mutant carrying the empty vector pCN34, and of the complemented mutant *secDF* pCQ27 in LB broth at 37°C and 15°C, respectively.

susceptibility to the β-lactam oxacillin and the glycopeptide vancomycin was observed (Figure 3). The reduction of oxacillin resistance was even more apparent in the presence of *mecA*, the gene encoding the penicillin binding protein 2a (PBP2a), mediating methicillin resistance, as shown for the methicillin resistant *S. aureus* (MRSA) strain pair Newman pME2 and Newman *secDF* pME2 (Figure 3) [28]. Reduction of oxacillin resistance in MRSA by *secDF* inactivation was confirmed in strains of different genetic backgrounds or SCC*mec* types, such as the clinical isolate CHE482 [29] and RA2 [30] or RA120 [31] (data not shown). The complementing plasmid pCQ27 was able to restore the wild type resistance levels.

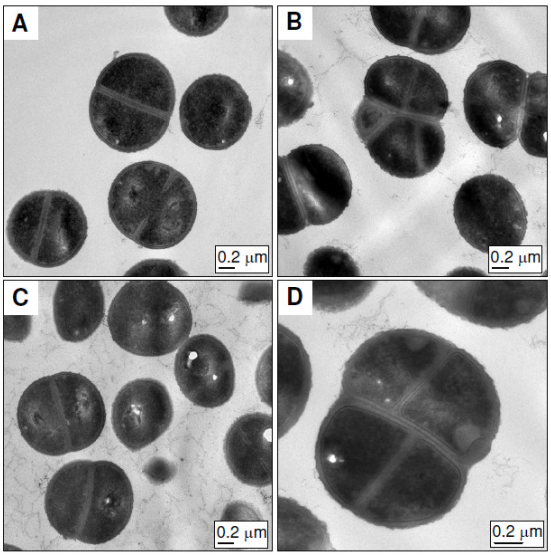

Figure 2 Cell morphology. TEM pictures from thin sections of strains **(A)** Newman pCN34, **(B and D)** Δ*secDF* pCN34 and **(C)** Δ*secDF* pCQ27 during exponential phase (OD_{600} 0.5).

Of the four endogenous *S. aureus* PBPs, PBP1 and PBP2 are essential, and reducing their expression lowers methicillin resistance even in the presence of the low β-lactam affinity PBP2a in MRSA [32,33]. As the Sec-system can promote protein insertion into the cytoplasmic membrane, we determined whether the reduced oxacillin resistance of the *secDF* mutant may be related to altered PBP amounts and/or subcellular localization. Staining cell membranes with the fluorescent penicillin-derivative Bocillin-FL [34] showed no major difference of PBP1-3 content in wild type MRSA background or corresponding *secDF* mutants (Figure 4A). However, Bocillin-FL staining did not allow the detection of the Sec-type signal peptide containing PBP4 [1] of approximately 48 kDa, or to distinguish the exogenous PBP2a in the Newman background (Figure 4A and 4B), possibly due to low protein levels or overlap, respectively. Western blots revealed comparable PBP2a and PBP4 amounts in the membrane fraction throughout growth, irrespective of the presence of SecDF (Figure 4B).

Increased autolysis and hydrolysis in the *secDF* mutant

Apart from functional PBPs, correct separation of daughter cells requires the controlled action of autolysins and hydrolases, many of which are Sec-dependent [1]. We therefore tested spontaneous and Triton X-100 induced autolysis to determine if the inability of *secDF* mutants to separate correctly was due to altered expression of autolytic activities. Both, spontaneous and Triton X-100 induced autolysis of the *secDF* mutant were increased in comparison to the wild type or the complemented mutant (Figure 5A).

To determine whether wild type and mutant bacteria produced different levels of hydrolases, their activity was

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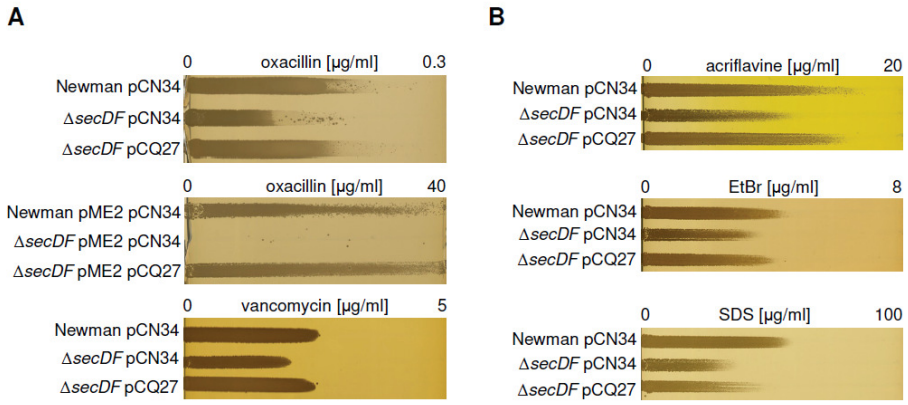

Figure 3 Effect of *secDF* inactivation on resistance profiles. **(A)** Gradient plates with increasing concentrations of β-lactam and glycopeptide antibiotics. Oxacillin was tested with methicillin sensitive and methicillin resistant strain Newman, the latter carrying the plasmid pME2 containing the *mecA* gene. **(B)** Gradient plates with increasing concentrations of the RND substrates acriflavine, ethidium bromide and SDS.

analyzed in concentrated supernatant and cell wall extracts (Figure 5B). In the supernatant of the mutant, high molecular mass bands matching different forms of the major *S. aureus* autolysin Atl [35], were expressed similarly (>130 kDa, pro-Atl) or even stronger (~84 kDa, PP-AM) compared to the wild type and the complemented mutant (Figure 5B). Interestingly, the >130 kDa band migrated at a slightly higher position in the mutant, corresponding to the height of the pro-Atl band in the cell wall fractions, where the mutant showed overall stronger hydrolytic bands than wild type or complemented mutant.

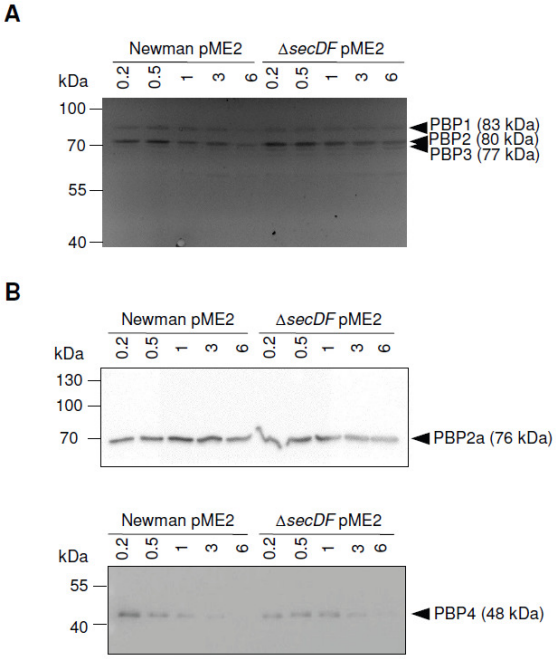

Figure 4 PBP expression over growth. Strain Newman pME2, carrying *mecA*, and its *secDF* mutant were cultivated in LB and samples collected at the indicated OD_{600} were used to prepare membrane fractions. **(A)** Membranes were incubated with the fluorescent penicillin analogue Bocillin-FL. Bands corresponding to PBPs 1-3 are indicated. **(B)** Western blot analysis of membrane fractions using antibodies against PBP2a and PBP4, respectively.

Deletion of *secDF* leads to altered expression of virulence factors

We qualitatively assessed the amount of various Sec-dependent *S. aureus* virulence factors, such as coagulase, hemolysin and protease activities, as well as of the immunomodulatory protein SpA to determine whether they were affected in the *secDF* mutant as well.

Supernatant from Newman and the complemented *secDF* mutant coagulated rabbit plasma after 30 min, whereas the *secDF* mutant required 90 min, suggesting production of slightly reduced coagulase levels. Extracellular proteolytic activity seemed to be absent in the *secDF* mutant, even after five days of incubation, whereas cultures from Newman and the complemented mutant showed distinct proteolytic halos on skim milk agar after 72 h (Figure 6A). Hemolysis activity was tested by a similar agar diffusion assay as used for protease activity determination. Overnight cultures, or sterile-filtrated culture supernatants, were dispensed into holes on sheep blood agar. Newman and the complemented *secDF* mutant showed the same extent of hemolysis. In the *secDF* background hemolysis was reduced when bacteria grew on the rim of agar holes (Figure 6B), but was increased when the hemolytic activity of sterile supernatant from liquid cultures was tested (Figure 6C and 6D). Sessile or planktonic growth affects regulatory mechanisms, which can alter the expression

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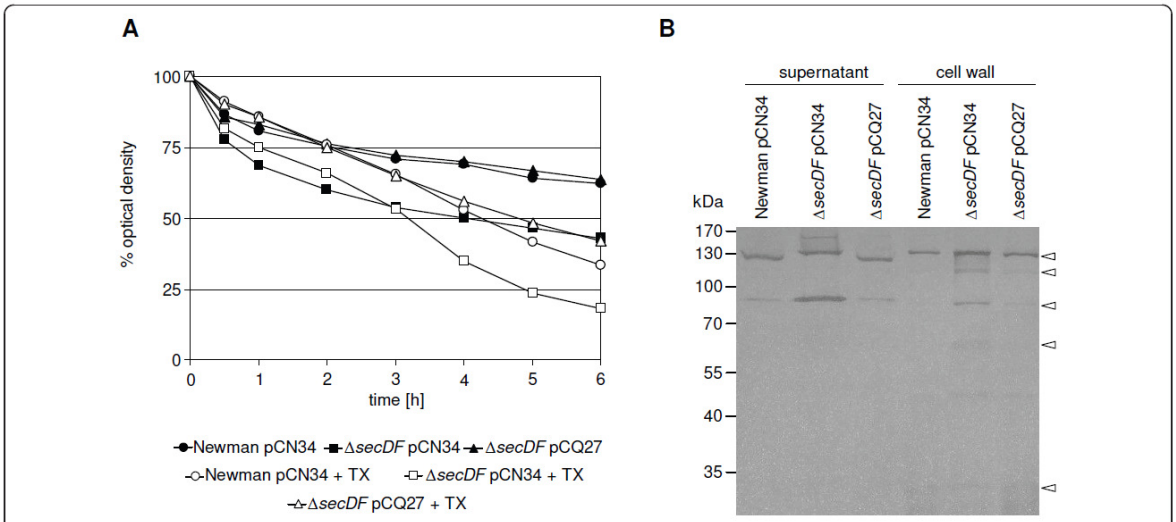

Figure 5 Autolysis and zymogram. **(A)** Spontaneous and Triton X-100 (TX) induced autolysis was measured over time. **(B)** Autolysin zymography of protein extracts from supernatant and cell wall was performed using SDS-10% PAGE supplemented with *S. aureus* cell wall extract as a substrate. Dark bands show hydrolyzed cell wall and are indicated by triangles. Based on the work of Schlag et al. bands were assigned as follows in decreasing order: Pro-Atl (~130 kDa); Atl (~115 kDa); Atl-amidase (~84 kDa) or part of the propeptide (62-65 kDa); Sle1/Aaa (~33 kDa) [35].

of virulence factors such as Hla [36,37]. Here we found that the deletion of *secDF* had divergent effects on hemolysin expression depending on the growth conditions, most likely by affecting regulatory processes.

SpA is one of the proteins predicted to be attached to the cell wall by sortase following export [38]. SpA levels were determined in subcellular fractions during growth by Western blot analyses.

Compared to the wild type, SpA levels were reduced in the cell wall and the cytoplasmic fraction, but slightly increased in the cell membrane fraction of the *secDF* mutant (Figure 7). The SpA levels were similar in the supernatant. Processed SpA has a molecular weight of approximately 51 kDa in strain Newman as estimated by Western blot analysis of wild type and Δ*spa* protein extracts (Additional file 1: Figure S1). Larger bands

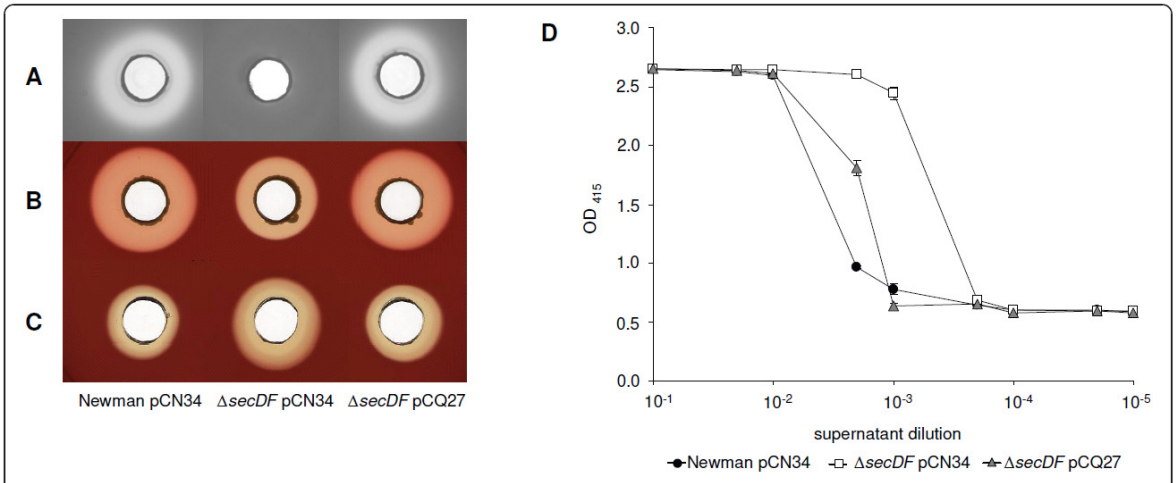

Figure 6 Proteolysis and hemolysis of sessile and planktonic bacteria. Proteolytic and hemolytic activity was determined qualitatively by agar diffusion assay on skim milk, respectively sheep blood agar. Hemolytic activity was measured in diluted sheep blood. **(A)** Skim milk agar and **(B)** sheep blood agar with sessile bacteria. **(C)** Sheep blood agar with sterile-filtered supernatants of stationary phase planktonic bacteria. **(D)** Release of hemoglobin by stationary phase supernatants of planktonic bacteria. Representative data of three independent experiments are shown with standard deviations of technical triplicates.

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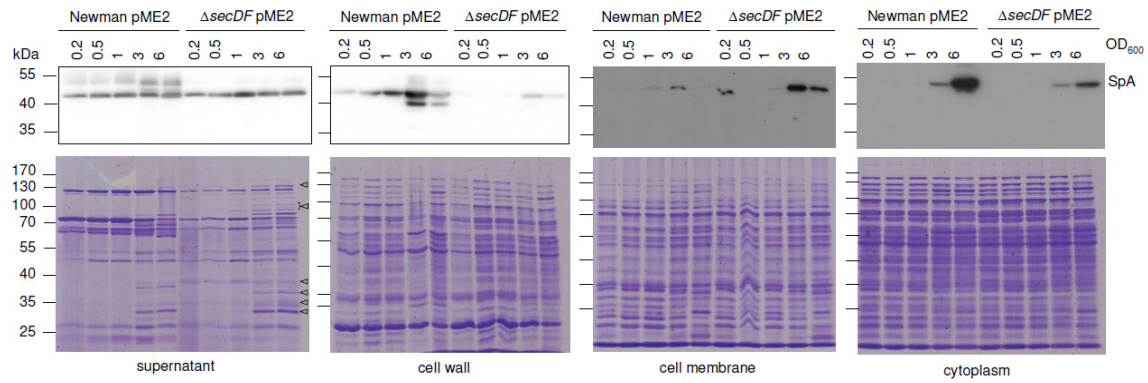

Figure 7 Subcellular localization of SpA. Expression and localization of SpA was monitored in the Newman pME2 background during growth. Upper panels show Western blots of SpA. Longer exposure times were required for detection of SpA in cell membrane and cytoplasm. Bottom panels show Coomassie stained gels. Bands of stronger expression in the mutant are indicated by triangles.

(~53 kDa) in the wild type supernatant fraction most likely represent SpA still attached to cell wall fragments. Thus, SpA translocation and/or processing seemed to be affected by the *secDF* deletion, a phenotype that could be complemented by introducing pCQ27 (data not shown).

Surprisingly, secreted SpA amounts were fairly constant despite this translocation defect. Also in the wild type, SpA levels in the supernatant were constant, whereas the amount of cell wall-bound SpA increased during growth, suggesting constant shedding of this protein.

Transcriptional analyses of virulence factors reveal regulatory changes in the *secDF* mutant

To determine whether the altered protein levels in the *secDF* mutant reflected also the mRNA level, transcription of *atl* (~3.8 kb), *coa* (~1.9 kb), *hla* (~1 kb) *hld* (~0.5 kb) and *spa* (~1.6 kb) were examined at different growth phases. *atl* transcription was elevated in the mutant during the entire growth (Figure 8) which is in agreement with the increased hydrolytic activities observed (Figure 5B). Transcription of *coa* sharply decreased after OD_{600} of 1. Slightly lower transcription levels were seen for *coa* in the *secDF* mutant (Figure 8), which is in line with our findings for its coagulation properties. As Newman carries a prophage in the *hlb* gene [39] and the gamma toxin is inhibited by sulfonated polymers in agar [40], we only looked at the transcription of the genes encoding α and δ toxins. *hla* amounts in the mutant were reduced compared to the wild type (Figure 8). The transcription pattern of *hld*, contained in the major regulatory RNAIII, had a tendency to being slightly reduced in the mutant but still showed a growth phase dependent expression, starting at OD_{600} 3 (Figure 8, data was assessed for the relevant ODs 1, 3 and 6). A striking difference was observed for the *spa* transcription, which in the wild type increased over growth with a peak at OD_{600} 3, but was drastically reduced in the *secDF* mutant (Figure 8). These findings were in contrast to the observed higher hemolytic activities and SpA amounts in the supernatant (Figure 6 respectively 7), which either suggests increased stability or reduced degradation of these proteins in the *secDF* mutant.

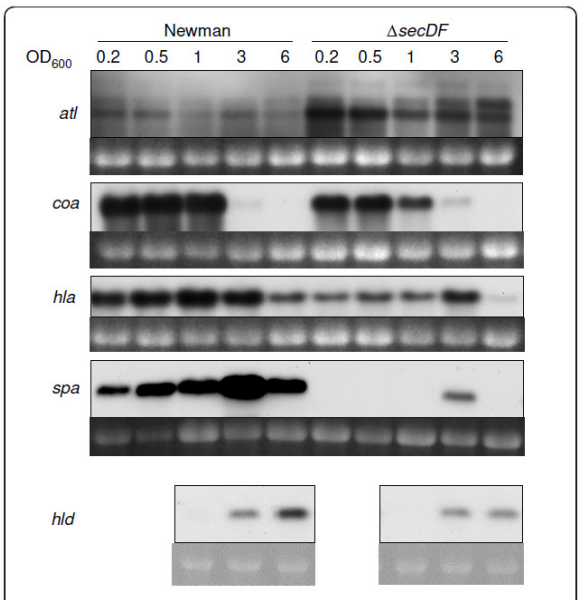

Figure 8 Transcription of virulence factors. *atl*, *coa*, *hla*, *spa* and *hld* transcription was monitored over growth in strains Newman and Δ*secDF*. Ethidium bromide-stained 16S rRNA is shown as an indication of RNA loading.

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Discussion

Efflux pumps play an important role in *S. aureus* resistance, virulence and pathogenicity. Yet the impact of the RND family of efflux pumps in staphylococcal resistance and fitness is still open (reviewed in [41]). To our knowledge, this is the first study to evaluate their role in *S. aureus*.

We found SecDF to contribute probably in part indirectly to resistance against several substances, including β-lactams and glycopeptides, making it an interesting target for increasing the efficacy of these standard antibiotics. In contrast Sa2056 and Sa2339 seemed not to be required for growth and resistance under the conditions tested. Banerjee et al. recently had found a conservative amino acid mutation in Sa2056 in a high-level β-lactam resistant *mecA*-negative strain [42]. However in that strain PBP4 and Sa0013 were also mutated and the exact reason for the observed resistance phenotype was not identified.

Resistance against cell wall active antibiotics and cell separation is dependent on a tightly balanced regulation of cell wall synthetic and hydrolytic enzymes, including their timely localization to the septum [43,44]. The amount of PBPs 1-4 and PBP2a was apparently not influenced, suggesting that other factors important for cell division and β-lactam resistance were affected. The increased hydrolytic activity in the *secDF* mutant may explain the observed differences in cell wall production and separation. Overproduction of a hydrolase has been observed to affect formation of the FtsZ-ring in *Mycobacterium tuberculosis* [45]. This cytoskeleton structure recruits the other cell division proteins to the site of future cell separation. A similar indirect effect in the *secDF* mutant might have lead to an incorrect localization of the cell division machinery, including PBPs (for a general review see [46]), thereby causing reduced resistance against the cell wall active antibiotics oxacillin and vancomycin. The difference in Atl processing might have impeded proper cell separation in addition.

Like *E. coli* and *B. subtilis secDF* mutants [6,24], the *S. aureus secDF* mutant displayed a cold-sensitive phenotype. In *E. coli* and *B. subtilis* SecDF has furthermore been shown to participate in membrane integration and secretion of proteins [6,24,47]. In *S. aureus* many physiological functions were affected by the *secDF* deletion. Analysis of the secretion of classical *S. aureus* virulence factors containing a Sec-type signal peptide revealed a complex picture. Coagulase and proteases were reduced in the supernatant in the *secDF* mutant. However, hemolysin activity under planktonic growth conditions was increased in the mutant, as was the case for (unprocessed) hydrolases, indicating that the *secDF* deletion did not lead to an overall reduction, but to an altered secretion and processing of proteins. In contrast hemolysin activity was reduced during sessile growth indicating that the deletion of *secDF* may have effects on overall metabolism.

SpA seemed to be impaired in reaching its destined subcellular localization. In the *secDF* mutant SpA accumulated in the membrane, was reduced in the cell wall fraction but was found in increased amounts in the supernatant. Altered secretion and processing of SpA might be due to impaired cell wall anchoring by the membrane protein sortase. However, Mazmanian et al. have shown that the extracellular enterotoxin B fused to the sorting signal of SpA accumulates in the cytoplasm and to a lesser extent in the membrane in a sortase mutant [48]. Thus, SpA might migrate by an alternate mechanism into the supernatant, circumventing linking to the peptidoglycan.

A similar divergent effect on protein secretion as we observed in the *secDF* mutant was found in a *secG* mutant. There SpA was found in increased amounts in the exoproteome, despite unaffected transcription [11]. In contrast, we found deletion of *secDF* to change mRNA levels for many of the analyzed genes, such as *atl*, *coa*, *hla*, *hld* and *spa*. The lack of *secDF* therefore seems to have a different impact on virulence factor expression than *secG*, influencing, most likely indirectly, transcription in addition to translocation. The absence of SecDF could especially cause a defective or reduced membrane insertion of sensor proteins belonging to one of the numerous *S. aureus* two component systems contributing to virulence factor regulation and to adaptations to different growth conditions (reviewed in [49,50]). The reduced *hld* levels in the mutant suggests that the *secDF* deletion affected at least one two component system by impairing signaling via the *agr* quorum sensor [51].

This study and the work of Sibbald et al. [11] once more demonstrate that protein and mRNA levels do not necessarily correlate. Specific regulation at the protein level has been shown for certain transcription factors in *S. aureus* [52,53]. Such a control of protein stability via chaperones and proteases might exist as well for virulence factors. Interestingly, in *E. coli*, *secY*, *yidC* and *secD* mutants were shown to induce the Cpx system, which up-regulates the expression of factors involved in folding and proteolysis in response to abnormal proteins in the outer membrane, the periplasmic space or the plasma membrane [54]. The induction of similar systems in the *S. aureus secDF* mutant due to clogging of the membrane, as suggested by the increased amounts of SpA in this compartment, could be an additional factor influencing protein stability and lead to the partially incoherent mRNA and protein levels, as seen for *hla*, *hld* and *spa* during planktonic growth.

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Conclusions

This work provides evidence that although *secDF* is dispensable in *S. aureus*, its deletion leads to a pleiotropic phenotype. Lack of SecDF affected cell separation, resistance and virulence factor expression showing that this conserved RND protein plays a major role in the important human pathogen *S. aureus*. Thus SecDF could be a potential therapeutic target rendering *S. aureus* more susceptible to the currently available antibiotics.

Methods

Bacterial strains and growth conditions

Strains and plasmids used in this study are listed in Table 1. Bacteria were grown aerobically at 37°C in Luria-Bertani broth (LB) (Difco) where not mentioned otherwise. Good aeration for liquid cultures was assured by vigorously shaking flasks with an air-to-liquid ratio of 4 to 1. Ampicillin 100 [μg/ml], anhydrotetracycline 0.2 [μg/ml], chloramphenicol 10 [μg/ml], kanamycin 50 [μg/ml] or tetracycline 10 [μg/ml] were added to the media when appropriate. Phage 80αalpha was used for transduction. Where nothing else is mentioned, experiments were repeated at least twice and representative data are shown.

Construction of mutants and complementation plasmid

In-frame markerless deletions of *sa2056* (NWMN 2'384'867-2'388'051), *sa2339* (NWMN 2'696'046-2'698'531) and *secDF* (NWMN 1'706'584-1'708'866) from the chromosome of *S. aureus* Newman (accession number NC_009641) was performed using pKOR1 [23] yielding single mutants CQ33, CQ65 and CQ66, respectively. Correct deletion was confirmed by PCR and by sequencing. Furthermore, strain stability was confirmed by pulsed field gel electrophoresis of total genome SmaI digests [55].

To complement the *secDF* mutant, *secDF* with its endogenous promoter was amplified from *S. aureus* strain Newman with primers listed in additional file 2 table S1.

Table 1 Strains and plasmids used in this study

Strain	Relevant genotype or phenotype	Ref. or source
S. aureus		
Newman	Clinical isolate (ATCC 25904), *rsbU*$^+$	[64]
RN4220	NCTC8325-4 r$^-$ m$^+$	[65]
CQ33	Newman*Δsa2056*	This study
CQ39	Newman pME2, Tc^r, Mc^r	This study
CQ65	Newman*Δsa2339*	This study
CQ66	Newman*ΔsecDF*	This study
CQ69	Newman*ΔsecDF* pME2, Tc^r, Mc^r	This study
CQ85	Newman pCN34, Km^r	This study
CQ86	Newman pCN34 pME2, Km^r, Tc^r, Mc^r	This study
CQ87	Newman*ΔsecDF* pCN34, Km^r	This study
CQ88	Newman*ΔsecDF* pCN34 pME2, Km^r, Tc^r, Mc^r	This study
CQ89	Newman*ΔsecDF* pCQ27, Km^r	This study
CQ90	Newman*ΔsecDF* pCQ27 pME2, Km^r, Tc^r, Mc^r	This study
E. coli		
DH5α	Cloning strain, [F-Φ80*lacZ*ΔM15 Δ(*lacZ*YA-*arg*F)U169 *rec*A1 *end*A1 *hsd*R17 (rk-, mk+) *pho*A *sup*E44 *thi*-1 *gyr*A96 *rel*A1 λ-]	Invitrogen

Plasmid	Relevant genotype or phenotype	Reference or source
pCN34	*S. aureus-E. coli* shuttle vector, pT181-*cop-wt repC aphA-3* ColE1 Km^r	[56]
pCQ27	pCN34 derivative carrying *secDF* and its promoter (Newman), Km^r	This study
pCQ30	pKOR1 derivative carrying 1 kb fragments of the region up- and downstream of *sa2056* amplified from Newman, ligated together with EcoRI and recombined at the *attP* sites, Ap^r, Cm^r	This study
pCQ31	pKOR1 derivative carrying 1 kb fragments of the region up- and downstream of *sa2339* amplified from Newman, ligated together with HindIII and recombined at the *attP* sites, Ap^r, Cm^r	This study
pCQ32	pKOR1 derivative carrying 1 kb fragments of the region up- and downstream of *secDF* amplified from Newman, ligated together with HindIII and recombined at the *attP* sites, Ap^r, Cm^r	This study
pKOR1	*E. coli-S. aureus* shuttle vector used to create markerless deletions; *repF*(Ts) *cat attP ccdB ori* ColE1 *bla* P_{xyl}/*tetO secY570*, Ap^r, Cm^r	[23]
pME2	pBUS1 derivative carrying *mecA* and its promoter (COLn), Tc^r, Mc^r	[28]

Abbreviations are as follows: Ap^r, ampicillin resistant; Cm^r, chloramphenicol resistant; Km^r, kanamycin resistant; Mc^r methicillin resistant; Tc^r, tetracycline resistant.

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The amplified region was ligated into the SalI/BamHI restriction sites of pCN34, a low copy (20-25 copies/cell) *E. coli-S. aureus* shuttle vector [56]. The junction region was sequenced as a control. The resulting plasmid pCQ27 was electroporated into RN4220 with subsequent transduction into the strains of interest.

To construct MRSA strains, the plasmid pME2, containing the *mecA* promoter and gene from strain COLn [28], was either electroporated or transduced into the strains selected.

Promoter predictions were performed by BPROM http://linux1.softberry.com/berry.phtml. Rho-independent transcriptional terminators were retrieved from the CMR terminator list http://cmr.jcvi.org/tigr-scripts/CMR/CmrHomePage.cgi.

Transmission electron microscopy (TEM)

Cells were grown to exponential phase, harvested at OD_{600} 0.5 and fixed for one hour in 2.5% glutaraldehyde in phosphate buffered saline (PBS) pH 7.4. Electron microscopy was performed by the Center for Microscopy and Image Analysis, University of Zurich.

Resistance profiles

For qualitative susceptibility comparisons, bacterial suspensions of McFarland 0.5 were swapped across LB agar plates containing antibiotic gradients and incubated at 35°C for 20-24 h. Glycopeptides were tested on Brain Heart Infusion (BHI) (Difco) agar with a bacterial suspension of McFarland 2 [57].

Spontaneous and Triton X-100 induced autolysis

Cells were grown to an OD_{600} of 0.7, pelleted by centrifugation and washed with 0.85% NaCl. The cells were then resuspended in 0.01 M Na-phosphate buffer pH 7 and the OD_{600} was adjusted to 0.7. After splitting the cultures, 0.01% Triton X-100 (Fluka) or an equal volume of PBS pH 7 was added. Cultures were incubated at 37° C and the decrease of OD_{600} was measured.

Zymographic analyses

Cultures were grown to an OD_{600} = 0.7, centrifuged and the filtered supernatants (pore size 0.45 μm, TPP) stored at - 20°C until further use. The cell wall peptidoglycan was digested in SMM buffer (0.5 M sucrose, 0.02 M maleate, 0.02 $MgCl_2$ pH 6.5) supplemented with 72 μg/ml lysostaphin and 2 mM phenylmethylsulfonyl fluoride (PMSF) [38]. Cell wall containing supernatant was separated from the protoplasts and stored at - 20°C until further use. Protein concentrations were measured by Bradford assay (BioRad).

Twenty μg of protein from each fraction was separated by SDS-10% polyacrylamide gel electrophoresis (PAGE) containing cell wall extract of heat-inactivated (1 hour at 100°C in 4% SDS) *S. aureus* (end concentration OD_{600} = 6). The gel was washed twice for 15 min in dH_2O and incubated for 18 h at 37°C in 0.1 M Na-phosphate buffer pH 6.8. Afterwards the gel was incubated for 3 min in staining solution (0.4% methylene blue, 0.01% KOH, 22% EtOH) and destained in cold water for several hours. Murein hydrolase activities produced clear bands.

Coagulase test

Overnight cultures were pelleted at full speed, 0.5 ml supernatant was transferred into fresh tubes and 2 mM PMSF was added. The supernatants were normalized to an OD_{600} of 1 of the original culture with PBS. 0.1 ml supernatant was added to 0.25 ml reconstituted rabbit plasma (BBL Coagulase Plasmas, BD) and incubated at 37°C. Every 30 min tubes were examined for coagulation.

Qualitative hemolysis assay

Cells were grown overnight in Todd-Hewitt (TH) medium [58], which was originally developed for the production of streptococcal hemolysins [59]. To visualize hemolysis production of sessile bacteria, overnight cultures were normalized to an OD_{600} = 1 in PBS pH 7.4. Fifty μl was dispensed into 5 mm wide holes punched into 5% sheep blood agar. Plates were incubated overnight at 37°C and then stored at 4°C. To determine hemolysis in liquid media, the overnight cultures grown in TH medium were normalized to the same OD_{600} with PBS and pelleted for 10 min at 5'900 g. The supernatant was filtered (pore size 0.22 μm, TPP) and 140 μl added to the holes in sheep blood agar. Plates were incubated as above.

Quantitative hemolytic activity

Cells were grown for 24 h in TH medium and normalized with PBS pH 7.4 to the same OD_{600}. After pelleting the cells, the filtered supernatants (pore size 0.22 μm, TPP) were diluted up to 1:50'000 in TH medium. Sterile sheep blood was treated with 26 mM sodium citrate and 15 mM NaCl and diluted 1:100 in PBS pH 7.4. After washing the erythrocytes four times in PBS pH 7.4, they were resuspended to a dilution of 1:100 in PBS pH 7.4. Five hundred μl of washed erythrocytes were added to 500 μl of the diluted supernatants and incubated for 30 min at 37°C, followed by 30 min at 4°C. Finally the samples were centrifuged for 1 min at 7'000 g and the absorption of hemoglobin in the supernatant was measured at 415 nm [58].

Determination of protease activity on skim milk agar plates

Skim milk agar plates were prepared as follows: Skim milk (Difco) and Bacto agar (Difco) were dissolved

separately in 250 ml dH_2O, each with an end concentration of 75 g/l and 15 g/l, respectively. After autoclaving for 15 min at 110°C and cooling down to 50°C, the skim milk and Bacto agar solutions were mixed together. Overnight cultures grown in LB broth were normalized to an OD_{600} = 1 with 0.85% NaCl and 50 µl was added into punched holes in skim milk agar. Skim milk agar plates were incubated at 37°C for 24 h and another 96 h at room temperature.

Transcription analyses

Prewarmed LB broth was inoculated with an overnight culture to an OD_{600} 0.05 and incubated at 37°C. Cells were harvested at OD_{600} 0.2, 0.5, 1, 3 and 6, centrifuged for 5 min at 20'000 g and 4°C. Cells were immediately snap frozen in liquid nitrogen and stored at - 80°C. Total RNA was extracted as described in [60]. Seven µg RNA was separated in a 1.5% agarose gel containing 20 mM guanidine thiocyanate in 1× TBE [61]. RNA was transferred onto a positively charged nylon membrane (Roche) using the downward capillary transfer method. The blots were hybridized with specific digoxigenin (DIG)-labeled DNA probes (Roche). Primers used are listed in Additional file 2 Table S1.

Analyses of subcellular protein fractions

Cells were sampled as described for transcription analyses and culture supernatant was collected as described for zymographic analysis. Cells were fractionated basically according to Schneewind et al. [38]. Briefly, cells were digested in SMM buffer supplemented with each 72 µg/ml lysostaphin and lysozyme, 36 µg/ml DNase and 2 mM PMSF. Protoplasts were separated from the cell wall containing supernatant by centrifugation for 4 min at 16'000 g. Protoplasts were resuspended in membrane buffer (0.1 M NaCl, 0.1 M Tris-HCl, 0.01 $MgCl_2$ pH 7.5) and lysed by three cycles of freezing in liquid nitrogen/thawing at 20°C. Cell membranes were separated from the cytoplasm by centrifugation for 30 min at 20'000 g and 4°C. Membrane pellets were solubilized in buffer B (25 mM Tris-HCl pH 7.5, 150 mM NaCl, 1 mM $MgCl_2$, 30% glycerol) supplemented with 1% Triton X-100 and 0.5% N-lauroylsarcosine, by gently mixing end-over-end at 4°C. Where necessary, protein fractions were concentrated with Amicon Ultra-15, -4 or -0.5 centrifugal filter units (MWCO 10 kDa, Millipore). Cell fractions were kept at - 20°C.

Five µg of protein was separated by SDS-10% PAGEs and either stained with Coomassie Imperial™ Protein Stain (Thermo Scientific) or blotted onto a PVDF-membrane (Immobilon-P, Millipore). For detection of SpA, membranes were blocked with 5% milk powder in PBS and then incubated with goat anti-human IgA conjugated with horseradish peroxidase (HRP, Sigma-Aldrich), 1:10'000 in 0.5% milk powder/PBS, 0.05% Tween 20 (AppliChem). After washing three times with PBS pH 7.4, HRP was detected with SuperSignal West Pico Chemiluminescent substrate (Thermo Scientific). PBP2a was detected as described in [28]. For detection of PBP4, membranes were blocked with 5% milk powder in PBS. Membranes were pre-incubated with 40 µg/ml human IgG in 0.5% milk powder/PBS. Rabbit anti-PBP4 antibodies (1:2000, [62]) and 0.05% Tween 20 were then added. After incubation for 1 h, membranes were washed three times with PBS before addition of goat anti-rabbit IgG-HRP (Jackson ImmunoResearch), 1:10'000 in 0.5% milk powder/PBS/0.05% Tween 20. After washing three times with PBS, HRP was detected as described for SpA. Molecular weights of PBP2a, PBP4 and unprocessed SpA are 76 kDa, 48 kDa and 56.7 kDa, respectively.

Bocillin-FL staining

Hundert µg of cell membrane fraction were incubated for 30 min at 35°C with Bocillin-FL (Invitrogen) as described by [63] before separation by SDS-7.5% PAGE. Fluorescence was visualized with the FluorChem™ SP imaging system (AlphaInnotech).

Additional material

Additional file 1: Figure S1 - SpA processing in strain Newman. Western blot analyses of **(A)** subcellular fractions of wild type grown to an OD_{600} of 3 and **(B)** of total extract from overnight cultures of wild type and *spa* mutant using goat anti-human IgA antibodies. Coomassie stained total protein is shown on the right as an indication of loading. SN, supernatant; CW, cell wall; CM, cell membrane; CP, cytoplasm.

Additional file 2: Table S1 - Primers used in this study.

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Authors' contributions

CQ carried out construction of strains, phenotypic characterizations, transcription analysis and drafted the manuscript. ASZ and RAS contributed to the growth condition experiments and participated in writing of the manuscript. MMS carried out the Western blot analyses, Bocillin-FL staining and participated in writing the manuscript. BBB coordinated the study and participated in writing of the manuscript. All authors read and approved the final manuscript.

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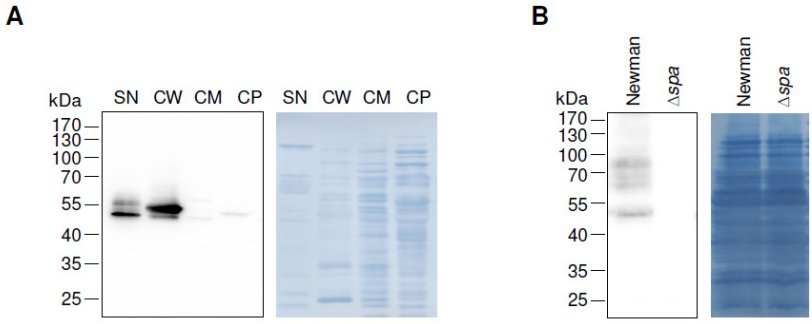

Figure S1 - SpA processing in strain Newman

Western blot analyses of **(A)** subcellular fractions of wild type grown to an OD_{600} of 3 and **(B)** of total extract from overnight cultures of wild type and *spa* mutant using goat anti-human IgA antibodies. Coomassie stained total protein is shown on the right as an indication of loading. SN, supernatant; CW, cell wall; CM, cell membrane; CP, cytoplasm.

Table S1: Primers used in this study

Primer	Sequence 5'-3'	Use	Reference
atl+	CCAAGGAACCATTGATAAGC	DIG labeling *atl*	This study
atl-	TGATACTGCTAAACCTACGC	DIG labeling *atl*	This study
CQ27	GCATCATTAGGTACAATCGTG	DIG labeling *sa2056*	This study
CQ31	AGTGTGGGGAACATACTTAAGTG	For sequencing junction Δ*sa2056*	This study
CQ61	TGCTTCGGCAATTAGTGTTG	DIG labeling *sa2339*	This study
CQ62	GCCCTTGCTTAGGTATCACG	DIG labeling *sa2339*	This study
CQ63	ATCGCCGCAGCAGTATTATT	DIG labeling *secDF*	This study
CQ65	GCACGCGTTAAATCGTCTTT	DIG labeling *secDF*	This study
CQ77	*GGGGACCACTTTGTACAAGAAAGCTGGGTC* CTGGTTATGCAATCGGCGGTTTG	pCQ32 construction	This study
CQ78	CACAAGCTTACCTCATTATTTACGTATGT	pCQ32 construction	This study
CQ81	*GGGGACCACTTTGTACAAGAAAGCTGGGTT* GCTTCATAATGAACAAGGG	pCQ31 construction	This study
CQ82	TTTAAGCTTAGTATATTGCCTCCTTTTAAA ATC	pCQ31 construction	This study

Table S1 continued.

Primer	Sequence 5'-3'	Use	Reference
CQ83	ATAAAGCTTAAAATAACATGTACATGCCT CCGC	pCQ31 construction	This study
CQ84	*GGGGACAAGTTTGTACAAAAAAGCAGGCT*GC TTGATACTTATCATGAGATG	pCQ31 construction	This study
CQ85	ATTAAGCTTTAAAATGAATTAAGCGGTAT GTGAAACAATAAAGAG	pCQ32 construction	This study
CQ86	*GGGGACAAGTTTGTACAAAAAAGCAGGCT*GC TAATGATGCGTCATCTAAACGACCTACAG C	pCQ32 construction	This study
CQ90	GAGGTCGGGGATAGATACT	For sequencing junction Δ*sa2339*	This study
CQ91	CTCCCTGCTCTAGTATGTT	For sequencing junction Δ*secDF*	This study
CQ98	ACTGGATCCTTTAAACTAAAATCTTTTCAT CGTTCG	*secDF* complementation	This study
CQ100	TCATTGCCGTTCCGCTATGG	For sequencing junction in pCQ27	This study
CQ101	GGGGACCACTTTGTACAAGAAAGCTGGGT CCTGATGTTGAAGTTGATAAAGG	pCQ30 construction	This study
CQ102	TCACGAATTCTCCCCCTCTTTCACTATATT C	pCQ30 construction	This study

Table S1 continued.

Primer	Sequence 5'-3'	Use	Reference
CQ103	TAATGAATTCGCCATAAAAGCGGTCATGA TATTG	pCQ30 construction	This study
CQ104	*GGGGACAAGTTTGTACAAAAAAGCAGGCT*CC ACAGATATTTTAGAAGTCTACTG	pCQ30 construction	This study
CQ105	GCAAATCAAGTCCTAAATTGAC	For sequencing junction in pCQ27	This study
CQ109	GGTGGTCGACAAGGTACTGTTAAAGCAG	*secDF* complementation	This study
CQ118	GGAGCACGCGAAAGAGTTACG	DIG labeling *coa*	This study
CQ119	CACGGATACCTGTACCAGCATC	DIG labeling *coa*	This study
MS20	AGAAAATGGCATGCACAAAAA	DIG labeling *hla*	[1]
MS21	TGTAGCGAAGTCTGGTGAAAA	DIG labeling *hla*	[1]
MS115	GGCTAGTCCTTTAACCTGTTTC	DIG labeling *sa2056*	This study
spaF	TGTAGGTATTGCATCTGTAA	DIG labeling *spa*	[2]
spaR	AAGTTAGGCATATTCAAGAT	DIG labeling *spa*	[2]

Restriction sites used for cloning are underlined. attB1 and attB2 sites written in italics.

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3.3 Project III

Secretome analysis defines the major role of SecDF in *Staphylococcus aureus* virulence

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Abstract

The Sec pathway plays a prominent role in protein export and membrane insertion, including the secretion of major bacterial virulence determinants. The accessory Sec constituent SecDF has been proposed to contribute to protein export. Deletion of *Staphylococcus aureus secDF* has previously been shown to reduce resistance, to alter cell separation and to change the expression of certain virulence factors. To analyse the impact of the *secDF* deletion in *S. aureus* on protein secretion, a quantitative secretome analysis was performed. Numerous Sec signal containing proteins involved in virulence were found to be decreased in the supernatant of the *secDF* mutant. However, two Sec-dependent hydrolases were increased in comparison to the wild type, suggesting additional indirect, regulatory effects to occur upon deletion of *secDF*. Adhesion, invasion and cytotoxicity of the *secDF* mutant were reduced in human umbilical vein endothelial cells. Virulence was significantly reduced using a *Galleria mellonella* insect model. Altogether, SecDF is a promising therapeutic target for controlling *S. aureus* infections.

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Introduction

The Gram-positive pathogen *Staphylococcus aureus* is one of the leading causes of nosocomial infections [1]. Due to its acquisition of various resistance genes treatment of *S. aureus* infections have become increasingly difficult. Furthermore, the prevalence of methicillin-resistant *S. aureus* (MRSA) has been increasing in recent years. This has resulted in an alarming rise of community-associated (CA-) MRSA infections in immunocompetent individuals [2,3,4]. In addition to its adaptive response to antibiotics [5], the success of *S. aureus* is based upon its huge array of virulence factors [6] helping *S. aureus* to avoid host immunity. These virulence factors have to be exported across the cytoplasmic membrane to reach their destined location: the membrane, the cell wall or the extracellular space. The main transport system is the Sec translocase, which is conserved in all three kingdoms of life [7,8]. Currently, the Sec pathway is best described in the Gram-negative bacterium *Escherichia coli* (as reviewed in [9,10]). The translocase consists of i) the heterotrimeric complex SecYEG, which forms a hydrophilic channel through the cytoplasmic membrane; ii) the motor protein SecA, an ATPase; and iii) the heterotrimeric complex SecDF-YajC. Proteins containing an N-terminal Sec signal peptide (SP) or a hydrophobic transmembrane segment are targeted to the translocase and transported through the channel in an unfolded state. For secreted proteins or membrane proteins with large hydrophilic loops, the driving energy is provided by the cycling of SecA, whereas ribosome-bound nascent chains are targeted mainly by inner membrane proteins [11] and are co-translationally exported powered by the translating ribosome. Small membrane proteins can also be inserted by YidC in a Sec-independent manner [12]. The auxiliary complex SecDF-YajC was shown to associate with SecYEG [13] as well as with YidC and is therefore believed to act as the linking molecule between SecYEG and YidC during Sec-dependent membrane protein insertion [12]. The integral membrane protein YajC was found to co-crystallize with the well-known *E. coli* multidrug exporter AcrB [14], which belongs to the resistance-nodulation-cell division (RND) superfamily. Deletion of YajC only showed a weak phenotype and its exact function is still unknown [15,16].

SecDF also belongs to the RND superfamily and possesses the typical twelve transmembrane (TM) domains with two extracytoplasmic loops between TM1-2 and TM7-8, respectively [17]. Recently, Tsukazaki *et al.* resolved the crystal structure of the membrane protein SecDF of *Thermus thermophilus* [18]. Two conformations for the head subdomain P1 are observed, that seem to occur upon rotation by 120° [18]. Furthermore the P1 head was shown to interact with an unfolded preprotein, thereby preventing the substrate from backsliding and enhancing the translocation. This ATP-independent step in the later stage of protein translocation is

driven by the proton motive force (PMF). Two conserved charged residues Asp519 and Arg247 are crucial for SecDF activity in *E. coli* and point mutations of the corresponding amino acids in *T. thermophilus* abolish ion channel activity [18].

In *S. aureus*, SecA and SecY have been shown to be essential [19,20,21]. Deletion of the non-essential *secG* leads to changes in the exoproteome, which are enhanced in a *secG-secY2* double mutant [7]. SecY2 together with SecA2 belong to the accessory Sec pathway, which at present is known to export only one substrate, the serine-rich adhesin for platelets protein (SraP) [22]. However, virulence of the *secG* and *secY2* single mutants and the *secG-secY2* double mutant in mice is comparable to the parental strain [7].

We previously reported a *S. aureus secDF* mutant to have a pleiotropic phenotype influencing not only protein secretion, but also transcription and regulatory processes [23]. Resistance towards β-lactam and glycopeptide antibiotics was reduced. Furthermore, cell division was impaired and autolysis increased [23]. To determine the role of SecDF in the secretion of virulence factors and to assess its importance for pathogenesis, we performed a secretome analysis using isobaric tags for relative and absolute quantitation (iTRAQ) with subsequent LC-MS/MS. Major virulence determinants involved in adhesion to host proteins and cells, as well as in evasion of the host immune system were found to be decreased in the exoproteome of the *secDF* mutant. Important steps for establishing an infection were shown to be deficient *in vitro* in the *secDF* mutant in both methicillin sensitive and resistant *S. aureus* strains. Furthermore, *secDF* virulence was significantly reduced in a *Galleria mellonella* infection model.

Materials and Methods

Bacterial strains and growth conditions

Bacterial strains and plasmids used in this study are listed in Table 1. If not mentioned otherwise bacterial cultures were grown in Luria Bertani (LB) broth (Becton Dickinson, Difco Laboratories, Franklin Lakes (NJ), USA) at 37 °C. Bacterial cultures were grown under constant shaking and with a liquid-to-air ratio of 1:5 to assure good aeration. Media were supplemented with 50 µg/ml kanamycin (Sigma-Aldrich, St. Louis (MO), USA) or 10 µg/ml erythromycin (Sigma-Aldrich, St. Louis (MO), USA) when appropriate.

Table 1. Strains and plasmids used in this study.

Strains	Properties	Ref.
S. aureus		
CHE482	CC45, ST45, SCC*mec*$_{N1}$, *blaZ* (pBla), Fa^r, Mc^r, Sx^r, Tm^r	[24,25]
Cowan I	NCTC8530, septic arthritis isolate	ATCC12598
CQ66	Newman Δ*secDF*	[23]
CQ85	Newman pCN34, Km^r	[23]
CQ87	Newman Δ*secDF* pCN34, Km^r	[23]
CQ89	Newman Δ*secDF* pCQ27, Km^r	[23]
CQ92	CHE482 pCN34, Km^r	This study
CQ93	ME305 pCN34, Em^r, Fa^r, Km^r, Mc^r, Sx^r, Tm^r	This study
CQ94	ME305 pCQ27, Em^r, Fa^r, Km^r, Mc^r, Sx^r, Tm^r	This study
ME305	CHE482 Δ*secDF*::*bursa aurealis*, Em^r, Fa^r, Mc^r, Sx^r, Tm^r	[26]
Newman	Clinical isolate (ATCC 25904), *rsbU+*	[27]
Plasmids		
pCN34	*S. aureus-E. coli* shuttle vector, pT181-*cop-wt repC aphA-3* ColE1, Km^r, called pEmpty in this study	[28]
pCQ27	pCN34 derivative carrying *secDF* with its endogenous promoter, Km^r, called pSecDF in this study	[23]

Abbreviations are as follows: Em^r, erythromycin resistant; Fa^r, fusidic acid resistant; Km^r, kanamycin resistant; Mc^r, methicillin resistant; Sx^r, sulfomethoxazole resistant; Tm^r, tobramycin resistant.

Sample preparation for secretome analysis

Strains were cultured until late exponential phase, which was shown to correspond to OD_{600} 1 in an earlier study [23] and centrifuged at 4 °C for 5 min. The supernatant (SN) was filtered (0.22 µM PES filter, Techno Plastic Products AG, Trasadingen, Switzerland) and mini EDTA-free complete protease inhibitors tablets (Roche, Rotkreuz, Switzerland) were added. For normalization purposes 500 pM enhanced GFP (Ams Biotechnology Ltd, Abingdon, UK) was added. The SN was concentrated by trichloroacetic acid (TCA) precipitation and washed

twice with ice cold (-20 °C) acetone. The pellet was resuspended in 0.5 M triethylammonium bicarbonate pH 8.5 (Sigma-Aldrich, St. Louis, USA) 0.5 mM tris(2-carboxyethyl)phosphine hydrochloride (TCEP, Sigma-Aldrich, St. Louis (MO), USA). Protein concentration was measured with the Quant-iT™ protein assay kit (Life Technologies, Invitrogen, Carlsbad (CA), USA).

Proteins were digested and labelled according to the iTRAQ protocol (AB Sciex, Concord, Canada). Briefly, 75 µg protein were denatured with 0.1 % SDS and reduced with 5 mM TCEP. 10 mM methyl methanethiosulfonate (MMTS) was used as a cysteine blocking reagent. Protein samples were digested with trypsin (Promega, Fitchburg (WI), USA) for 14.5 h at 37 °C and subsequently labelled with a 4-plex-iTRAQ Reagent for 1.5 h at room temperature. Phosphoric acid was added to stop the reaction and samples were combined into a fresh Eppendorf tube. Peptides were fractioned by strong cation exchange (SCX). Solvent A (7 mM KH_2PO_4, 25 % acetonitrile (ACN), pH 2.7) was added to the sample and loaded onto a polysulfoethyl A column (200 x 2.1 mm, 5 µm, 200 Å, PolyLC) of the analytical HPLC (LC1100, Agilent Technologies, Santa Clara (CA), USA). Peptides were eluted with an increasing gradient of solvent B (7 mM KH_2PO_4, 0.5 M KCl, 25 % ACN, pH 2.7) 10-50 min, 0-30 % solvent B; 40-60 min, 30-100 % solvent B) and pooled into 12 fractions according to the chromatogram. Peptides were concentrated with the SpeedVac® (Eppendorf, Hamburg, Germany), redissolved in 3 % ACN, 0.1 % trifluoroacetic acid and desalted using ZipTips C_{18} (Merck Millipore, Billerica (MA), USA). After a further vacuum concentration step the peptides were dissolved in 3 % ACN, 0.1 % formic acid (FA).

Mass spectrometry

Dissolved samples were injected into an Eksigent-nano-HPLC system (Eksigent Technologies, Dublin (CA), USA) by an auto sampler and separated on a self-made reverse-phase tip column (200 µm x 150 mm) packed with C_{18} material (3 µm, 200 Å, AG, Bischoff GmbH, Leonberg, Germany). The column was equilibrated with 99 % solvent A (1 % ACN, 0.2 % FA in water) and 1 % solvent B (0.2 % FA in ACN). Peptides were eluted using the following gradient: 0-3 min; 1-5 % B, 3-57 min; 5-35 % B, 57-63 min; 35-50 % B and 63-70 min; 50-99 % B, at a flow rate of 0.7 µl/min. High accuracy mass spectra were acquired with an AB Sciex 5600 (AB Sciex, Concord, Canada) in the mass range of 400-1250 m/z. Up to 40 data dependent MS/MS were recorded in high sensitivity mode of the most intense ions with charge state 2+, 3+ and 4+ using collision induced dissociation. Target ions already selected for MS/MS were dynamically excluded for 90 s after tree accuracies. After data collection,

the peak lists were generated and analyzed using ProteinPilot™ 4.0 (AB Sciex, Concord, Canada). Data was searched against a SwissProt database (released January 2011). The following search parameters were used: Trypsin digestion, modifications of MMTS labelled cysteine, 4-plex-iTRAQ modifications of free amines at the N-termini and of lysine, and 4-plex-iTRAQ modifications of tyrosine. Biological modifications and single amino acid exchanges were also included in the search. No normalization for iTRAQ ratios, such as BIAS correction, was applied, as normalization was performed by the addition of extrinsic GFP. The peptides without any 4-plex-iTRAQ label at the N-terminus or at a lysine were excluded from the analysis. The ProteinPilot cut-off score used was 1.3, which corresponds to a confidence limit of 95 %. In total four biological replicates were analyzed in two independent iTRAQ experiments. The statistical analysis was assessed by the Student's *t* test.

SDS-PAGE and Western blot

The SN was collected using the secretome sample preparation method and concentrated with Amicon Ultra-15 centrifugal filter devices (MWCO 10 kDa Merck Millipore, Billerica (MA), USA). Ten to 20 µg of protein were separated by SDS (10-15 %) polyacrylamide gel electrophoresis (PAGE) and blotted onto polyvinylidene fluoride (PVDF) membrane (Immobilon-P, Merck Millipore, Billerica (MA), USA). Blocking and detection were performed in phosphate buffered saline (PBS) pH 7.4 (CHIPS, FnBPA) or in low salt buffer (0.154 M NaCl, 9 mM TRIS, 0.1 % Tween) pH 7.4 (Eap, LytM, SceD and SEA) as described in [23]. The following primary antibodies were used: Mouse anti-CHIPS antibody (Abcam, Cambridge, UK) 1:1'000, rabbit anti-Eap antibody (Abcam, Cambridge, UK) 1:5'000, rabbit anti-FnBPA antibody (Abnova, Taipei City, Taiwan) 1:2'500, rabbit anti-LytM antibody (obtained from T. Msadek [29]) 1:50'000, rabbit anti-SceD antibody (obtained from S. Foster [30]) 1:10'000 and rabbit anti-SEA antibody (Abcam, Cambridge, UK) 1:1'000. For detection of the primary antibodies either horseradish peroxidase- (HRP-) goat anti-rabbit IgG (1:10'000, Jackson ImmunoResearch, West Grove (PA), USA) or HRP-goat anti-mouse IgG (1:5'000, Jackson ImmunoResearch, West Grove (PA), USA) were used.

Fibrinogen- and fibronectin-binding assay

The binding assay was adapted from O'Neill *et al.* [31]. Shortly, 100 µl two-fold dilutions of human fibrinogen (Merck, Calbiochem, Darmstadt, Germany) in 20 mM sodium citrate-HCl, pH 7.4 or human fibronectin (Merck, Calbiochem, Darmstadt, Germany) in PBS pH 7.4 were dispensed in flat-bottom 96-well Nunclon plates and incubated overnight at 4 °C. Following

three PBS wash steps, the plates were blocked in 2 mg/ml bovine serum albumin (BSA, Thermo Scientific, Acros Organics, Geel, Belgium) in PBS for 2 h at 37 °C. Wells were washed three times in PBS. In the meantime bacterial cultures were grown to OD_{600} 1, washed twice in PBS and adjusted to OD_{600} 1 in PBS corresponding to ~10^8 cells/ml. Hundred µl of bacterial suspension was added per well (10^7 CFU / well), including a negative control (PBS) and incubated for 2 h at 37 °C. After repeated PBS washing steps, the cells were fixed with 25 % formaldehyde (Applichem, Darmstadt, Germany), washed once with PBS and stained for 5 min in 0.5 % crystal violet. Residual dye was removed with distilled water (dH_2O) and plates were air-dried. The crystal violet stained cells were dissolved in 5 % acetic acid and the absorbance measured at 570 nm. The experiment was performed with three technical and biological replicates, except for the positive control strain Cowan I in the Newman fibronectin binding assay, which was only performed with three technical replicates.

Tissue culture

Human umbilical vein endothelial cells (HUVECs) were purchased from Clonetics (Lonza, Basel, Switzerland) and maintained as previously described in M199 medium supplemented with antibiotics (penicillin 100 U/ml, streptomycin 100 µg/ml), glutamine at 2 mM, and 20 % foetal calf serum, at 37 °C in a humidified 5 % CO_2 atmosphere until they reached confluency [32]. One day before assays were performed, HUVECs were seeded at a density of 5 x 10^4 cells/well into 48-well plates that were coated with 10 µg/ml fibronectin. Cells were used up to passage three.

Bacterial adherence and invasion to HUVECs

The capacity of the various strains to invade HUVECs was determined by the lysostaphin protection assay using conditions used for the MTT assays (see below) as previously described [33]. Adherence and invasion were expressed as % adherent and invading cells per well at the time point of measurement. Adherence and invasion of CHE482 were set to 100 % for each run. Each experiment was performed in duplicate in three independent assays.

MTT assay

The ability of *S. aureus* to induce damage in HUVECs was assessed using an MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay. MTT is reduced by living cells to insoluble purple MTT formazan crystals using succinate, and the pyridine nucleotide cofactors, NADH and NADPH as substrates [34]. MTT production is therefore inversely

related to cell death. MTT reduction results in a yellow to blue color change that can be quantified by measuring the absorbance at OD_{570} [35]. MTT assays were performed as previously described with some modifications [36]. Briefly, wells containing HUVECs were rinsed twice with warm HBSS prior inoculation to remove medium containing antibiotics. The bacterial strains were grown overnight on sheep blood agar, resuspended in invasion medium (1 % albumin and 25 mM HEPES, pH 7.3 in M199 without serum or antibiotics) to McFarland 0.5 (~5 x 10^7 cells/ml), sonicated and diluted to the indicated multiplicity of infection (MOIs) [37,38,39]. Initial inocula were confirmed by serial fold dilution and plating on sheep blood agar. After 1 h or 3 h incubation at 37 °C in 5 % CO_2, respectively, the number of CFUs per well was determined to monitor growth. Then, the medium was aspirated, wells were washed with HBSS and 400 µl of fresh complete M199 medium containing 10 µg/ml lysostaphin was added [33,38,40]. After a total incubation time of 22 h at 37 °C in 5 % CO_2, 100 µl of 5 mg/ml MTT in HBSS were added to each well. 2 h later, the medium was removed and 150 µl of 0.04 M HCl in absolute isopropanol were added to solubilize the dye. Uninfected control wells which underwent the same washes were processed in parallel and served as negative control. Wells that only contained medium were used as background correction. Absorbance of 100 µl of the solution was measured at 570 nm. Specific cytotoxicity was calculated using the following formula: 1 - (OD_{570} experimental well / OD_{570} control well). Each experiment was performed in triplicate in at least three independent assays.

Galleria mellonella virulence assay

To study the *in vivo* pathogenicity an invertebrate infection model was used as previously described by Peleg *et al.* [41]. Last instar larval stage *G. mellonella* were purchased at HRH Fishing Hebeisen, Zurich, Switzerland and stored at 4 °C until further use. Cells were grown until exponential phase (OD_{600} 0.5), washed twice in PBS and resuspended therein. Ten µl of bacterial suspension, corresponding to 10^6 CFUs, were injected into the last left proleg with a repetitive dispensing Tridak Stepper (Intertronic, Oxfordshire, UK) containing a 1 ml syringe with a 26-gauge needle. Thirty larvae were infected per strain. A control group was inoculated with 10 µl of PBS to assure the larvae were healthy and that death did not occur due to the needle prick or stress. Larvae were incubated at 37 °C and examined every 24 h for survival; they were considered dead when no movement occurred in response to touch. Additional larvae were inoculated separately to measure the bacterial burden which was monitored after 24, 48 and 72 hours. After incubating the larvae at -20 °C, they were disinfected in 70 %

ethanol and rinsed with dH_2O. The larvae were homogenized with the TissueLyser (Qiagen, Hombrechtikon, Switzerland) in 2-ml screw cap tubes containing a 5 mm stainless steel bead (Qiagen, Hombrechtikon, Switzerland) and 1 ml PBS, shaking for 10 min at 30 s^{-1}. Appropriate dilutions were plated on LB agar containing 50 µg/ml kanamycin to minimize growth of the normal flora of the larvae. Three independent experiments were pooled and analysed by log rank test. The survival-curves were plotted using the Kaplan-Meier method.

Results

Deletion of *secDF* leads to an altered exoproteome

To determine the extent of the extracellular proteome affected by deletion of *secDF*, a quantitative secretome analysis was performed for the *S. aureus* wild type strain Newman and its mutant strain Newman Δ*secDF* using iTRAQ. The SNs were collected during late exponential phase, where we found strongest SecDF expression (data not shown). Samples of four biological replicates were prepared for LC-MS/MS as described in materials and methods.

A total of 230 *S. aureus* proteins were quantified and their putative localization was determined with different bioinformatics tools (Table 2, Supplemental Table S1). Thirty-eight proteins had a predicted SP [42], 34 thereof were predicted Sec-type SPs [43]. These included seven cell wall proteins containing the LPXTG cell wall retention motif (SpA, SdrE, ClfA, IsdA, FnBPA, ClfB) [44,45] or LysM domains (Sle1/Aaa) [46]. In Newman Δ*secDF*, in comparison to the wild type Newman, the extracellular levels of 27 proteins were altered significantly by at least two fold increase (two proteins) or two fold decrease (25 proteins); 21 of these proteins contained a Sec-type SP (Figure 1). Of the six remaining proteins, three were membrane proteins; OatA and the LytR-CpsA-Psr proteins NWMN_0925 (SA0908) and MsrR [47,48]. Furthermore, the SP containing, but Sec-independent enterotoxin A (SEA) and the delta hemolysin (Hld) were identified.

According to their function the majority of the proteins were classified into three groups: i) Proteins with adhesive properties (ClfA, ClfB, Coa, Emp, Efb/Fib, FnBPA, Eap/Map, SpA) [49,50]; ii) proteins which are involved in immune evasion (CHIPS, ClfA, Efb, FLIPr, Eap, OatA, Sbi, SpA, SCIN, SEA, SspB) [51,52] and iii) autolytic proteins (IsaA, LytM, SceD, SsaA) [30,53,54]. The autolysin LytM and the lytic transglycosylase SceD were the only proteins found to be significantly increased in the secretome of Newman Δ*secDF*. Furthermore, several proteases were decreased in Newman Δ*secDF*, such as SspB, SspA and ScpA, whereof the amounts of SspB and ScpA were significantly reduced.

Peptides of the N-terminal region of the glycerol ester hydrolase Geh were identified and found to be reduced in Newman Δ*secDF*. Because one of the four prophages in strain Newman is located in the Geh gene *geh*, leading to a truncated and inactive enzyme [55], this finding is not phenotypically relevant in the strain Newman background. Nonetheless, in other *S. aureus* strain backgrounds, deletion of *secDF* can be expected to reduce lipase activity.

Table 2. Localization of identified proteins based on different bioinformatics tools [56,57,58] and Sibbald *et al.* [43].

Localization	Proteins (%)	Sec signal peptide	Remarks
Cytoplasm	172 (74.8)		
Membrane	9 (3.9)		
Lipoprotein	2 (0.9)		
Cell wall	7 (3.0)	7	6 covalently attached by LPXTG motif
Extracellular	31 (13.5)	28	6 non-covalently bound to the cell wall
Unknown	9 (3.9)		
Total	230 (100)		

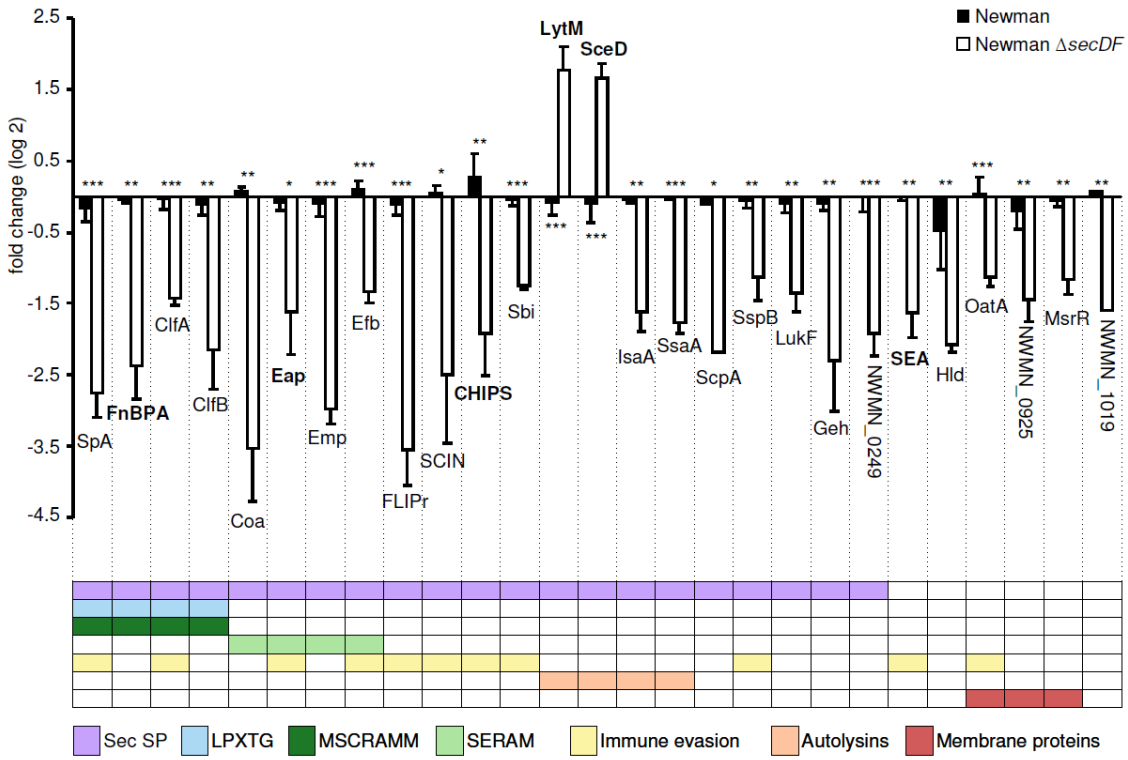

Figure 1. Differential extracellular protein amounts in Newman Δ*secDF* in comparison to the wild type Newman. Proteins identified to be significantly and more than two-fold changed in Newman Δ*secDF* compared to the wild type Newman. The mean values of four independent experiments are shown with their standard deviation given, except for NWMN_1019 and ScpA, which were only found in two biological replicates. Proteins are colour coded according to the following categories: N-terminal Sec signal peptide (SP), LPXTG-motif, adhesive properties (MSCRAMM and SERAM), immune evasive properties, autolytic properties and membrane proteins. Proteins confirmed below by Western blot analysis are highlighted in bold. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

Complementation of the altered virulence factor expression in the mutant strain Newman Δ*secDF* and verification of its phenotype in a second strain background

To validate and further highlight the role of SecDF in *S. aureus*, a different, methicillin resistant strain background was included in the following confirmatory experiments. The low level methicillin resistant strain CHE482 represents a CA-MRSA [24,25,59]; its corresponding *secDF* mutant CHE482 Δ*secDF* carries a transposon at position 1029 bp leading to a non-functional SecDF protein [26]. To complement the deletion of *secDF*, the plasmid pSecDF (pCQ27) containing *secDF* with its endogenous promoter from strain Newman was introduced into the mutant strains Newman Δ*secDF* and CHE482 Δ*secDF*, yielding Newman Δ*secDF* pSecDF and CHE482 Δ*secDF* pSecDF, respectively. The empty vector pEmpty (pCN34) was introduced into the wild type strains Newman and CHE482 and the *secDF* mutants Newman Δ*secDF* and CHE482 Δ*secDF* to ensure no additional effects were caused by the plasmid (Table 1).

To confirm the secretomics results, Western blot analysis of the SN from these strains were performed for selected proteins found to be altered in the secretome of Newman Δ*secDF*. Specific antibodies were used for the Sec-dependent proteins CHIPS, Eap, FnBPA, LytM and SceD, as well as the Sec-independent SEA.

As expected, the SN of Newman Δ*secDF* showed reduced protein amounts of CHIPS, FnBPA, Eap and SEA and increased amounts of LytM and SceD in comparison to the wild type and the complemented mutant (Figure 2A). This phenotype was validated and confirmed for CHIPS, FnBPA and SceD in strain background CHE482 (Figure 2B).

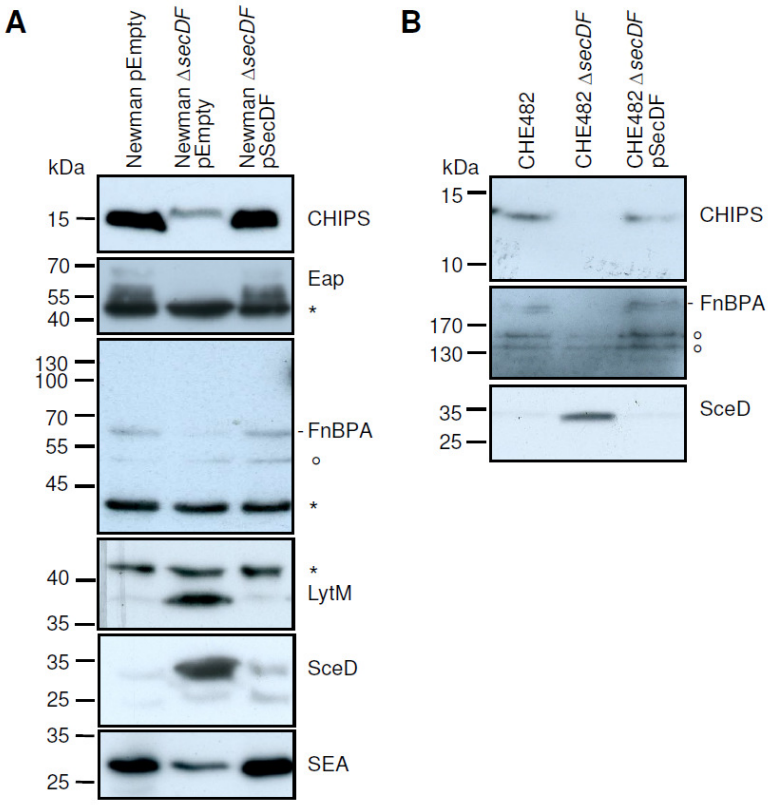

Figure 2. Complementation of SecDF-dependent changes. Western blot analysis of extracellular proteins in the SN of two different strain backgrounds. (A) Newman harbouring empty plasmid pEmpty (pCN34), the mutant strain Newman Δ*secDF* harbouring empty plasmid pEmpty (pCN34) and the complemented mutant Newman Δ*secDF* mutant harbouring plasmid pSecDF (pCQ27) containing the *secDF* gene with its endogenous promoter. (B) CHE482, the mutant strain CHE482 Δ*secDF* and the complemented mutant strain CHE482 Δ*secDF* harbouring plasmid pSecDF (pCQ27). Specific antibodies against CHIPS, FnBPA, Eap, LytM, SceD and SEA were used. Truncated FnBPA in Newman runs below the 70 kDa marker band. Putative degradation bands of FnBPA have been observed before [60,61,62,63] and are indicated by a ring. Additional protein bands due to unspecific binding to protein A or Sbi are indicated by an asterisk.

Decreased adherence of the *secDF* mutant to attached human fibrinogen, fibronectin and endothelial cells

To establish an infection, the ability of *S. aureus* to adhere to host proteins and cells is essential and permits the bacteria to invade into the cells as shown previously for *S. aureus* [64]. Our secretomics screen revealed several factors belonging to the microbial surface components recognizing adhesive matrix molecules (MSCRAMM) or the secretable expanded repertoire adhesive molecules (SERAM) to be reduced in Newman Δ*secDF* (Table 3). Therefore, binding of the *secDF* mutants to immobilized human fibrinogen and fibronectin, respectively, was studied *in vitro*. Ninety-six-well-plates were coated with

various concentrations of human fibrinogen and fibronectin, respectively. Bacteria were allowed to adhere to the coated wells, washed, fixed and coloured with crystal violet as described in materials and methods.

Binding of Newman Δ*secDF* pEmpty to fibrinogen was reduced by up to 50 % in comparison to the wild type strain Newman pEmpty and the complemented mutant Newman Δ*secDF* pSecDF (Figure 3A). In CHE482, binding of CHE482 Δ*secDF* pEmpty was reduced at low concentrations of fibrinogen, but not at concentrations above 2 µg/ml (Figure 3B). This phenotype was restored in both strains by the complementing plasmid pSecDF.

Fibronectin binding proteins are not anchored to the cell wall in strain Newman due to a point mutation leading to an early stop codon and truncated proteins without sortase motif [61]. Therefore, strain Cowan I was used as a functional control in the binding assay of Newman to human fibronectin [61]. As expected, the binding capacity of strain Newman pEmpty to fibronectin was up to ten times lower as compared to strain Cowan I (Figure 3A). However, deletion of *secDF* further reduced binding to fibronectin in the Newman background and was partially restored by the complementing plasmid. In CHE482, the *secDF* mutant showed a similar concentration dependent phenotype for fibronectin as for fibrinogen, with the CHE482 Δ*secDF* pEmpty displaying a reduced binding at lower fibronectin concentrations (Figure 3B). These findings suggest the presence of additional fibrinogen and fibronectin binding proteins in the CHE482 background, possibly on mobile genetic elements, which reduce the effect of a *secDF* deletion on adhesion to fibrinogen and fibronectin.

In a next step, we investigated whether the *secDF* deletion would also affect adherence to host endothelial cells (human umbilical vein endothelial cells, HUVECs). Because of the point mutation in the FnBPs of strain Newman mentioned above, this strain has been previously shown to be weakly adherent to HUVECs [61]. Therefore, we used the *secDF* mutant of strain CHE482 to test adherence and found significantly reduced adherence as compared to the wild type strain (Figure 4A). This effect was restored in the complemented mutant CHE482 Δ*secDF* pSecDF. In accordance with the previous findings [61] adherence by strain Newman was only 12.7 ± 6.6 % of strain CH482.

Table 3. Adhesins, which were reduced in the SN of Newman Δ*secDF*.

	Proteins	Binding to human fibrinogen	Binding to human fibronectin	Ref.
MSCRAMM	ClfA	x		[65]
	ClfB	x		[66]
	FnBPA	x	x	[60,67]
	SpA			
SERAM	Coa	x		[68]
	Eap	x	x	[69]
	Emp	x	x	[70]
	Efb	x		[71]

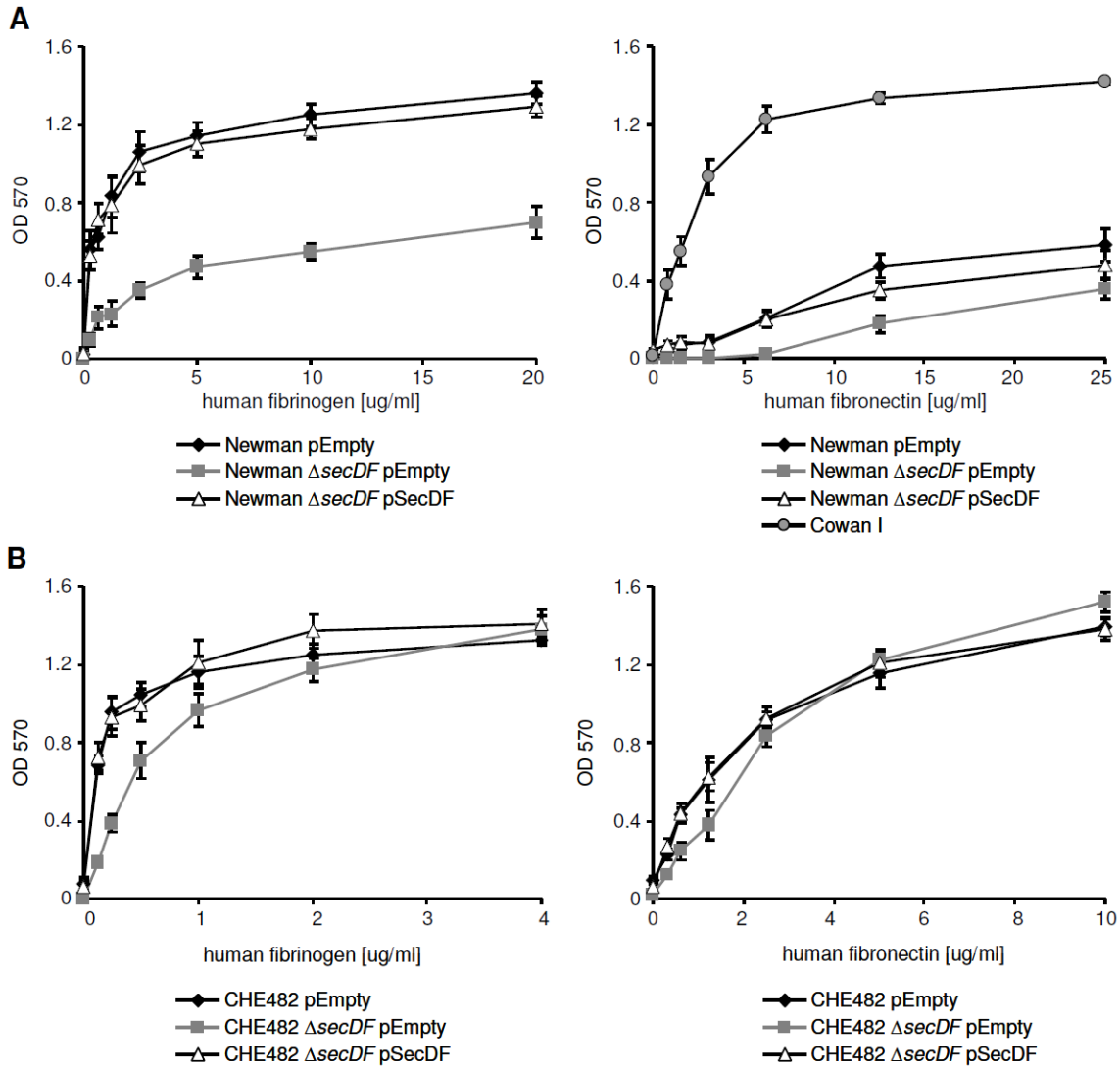

Figure 3. Impact of SecDF on fibrinogen and fibronectin binding. Binding properties of Newman and CHE482 strain sets to immobilized human fibrinogen and fibronectin was assessed as described in materials and methods. (A) Newman pEmpty, Newman Δ*secDF* pEmpty and the complemented mutant Newman Δ*secDF* pSecDF. FnBPs in the Newman background are truncated due to a point

mutation leading to a stop codon before the sortase motif, which is required for cell wall anchoring, and therefore are secreted [61]. Hence, Cowan I was used as a functional control strain in the fibronectin binding assay. (B) CHE482 harbouring empty plasmid pEmpty (pCN34), the mutant strain CHE482 Δ*secDF* harbouring empty plasmid pEmpty (pCN34) and the complemented mutant CHE482 Δ*secDF* pSecDF. Mean of three independent experiments are shown with their standard deviation.

secDF inactivation leads to reduced cytotoxicity

Many of the virulence factors produced by *S. aureus* destroy host tissue to allow dissemination. Thus, we analyzed the ability of the *secDF* mutant to damage endothelial cells using a previously published MTT assay [36]. This assay measures MTT reduction by living cells and is inversely related to cell death. We previously showed that MTT reduction by HUVECs internalized *S. aureus* is negligible [36]. To evaluate cytotoxicity of strain Newman, we used similar conditions as previously described using a MOI of 50 [38]. In this assay, endothelial cells are infected with bacteria for 3 h. Then, extracellular bacteria are killed and damage is assessed 24 h after the addition of the bacteria. Strain Newman did not induce substantial damage under these conditions (15.8 ± 10.9 % damage) and was not suited to study the effect of *secDF* inactivation on *S. aureus* cytotoxicity. We therefore decided to use strain CHE482, which caused a significant higher damage (69 ± 15 %) and the corresponding *secDF* mutant. However, CHE482 Δ*secDF* exhibited a growth defect in invasion medium during the 3 h invasion step (Figure 4B). We therefore decreased the invasion time to 1 h, while bacteria appeared to be in the lag-phase showing no measurable difference in OD_{600} or CFUs between the strains (Figure 4B). Because these conditions have not been previously used to study *S. aureus*-induced endothelial cell damage, it was essential to identify an optimal MOI. Figure 4C shows the increasing cytotoxicity of strain CHE482 with increasing MOIs ranging from 25 to 2500. Again, damage induced by strain Newman under these conditions was very low. Using an MOI of 2500, we found that *secDF* inactivation in strain CHE482 led to a substantial reduction in cytotoxicity (Figure 4D). This phenotype could be complemented using strain CHE482 Δ*secDF* pSecDF, which contains the *secDF* locus on a plasmid. To determine whether the reduced cytotoxicity of CHE482 Δ*secDF* was due to a reduction in endothelial cell invasion, invasion was assessed under similar conditions as for the MTT assay. The mutant strain CHE482 Δ*secDF* exhibited significantly reduced invasion as compared to the wild type strain (Figure 4E). This effect was restored in the complemented mutant CHE482 Δ*secDF* pSecDF. In accordance with the low adherence and cytotoxicity of strain Newman, invasion was only 1.6 ± 1.0 % of strain CHE482.

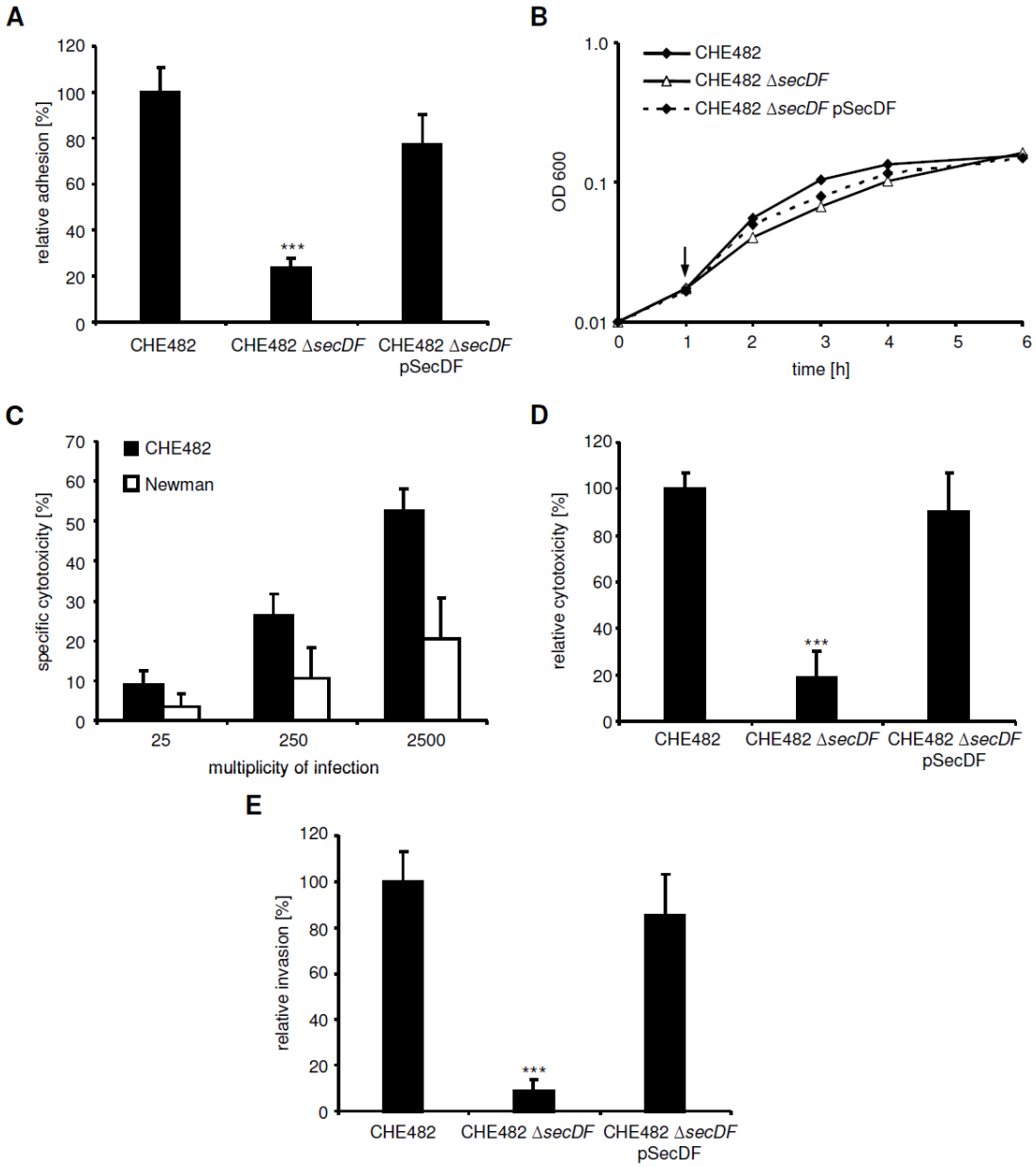

Figure 4. Adhesion, cytotoxicity and invasion in HUVECs. Interactions of Newman and the CHE482 strain set the wild type CHE482, CHE482 Δ*secDF* and the complemented mutant CHE482 Δ*secDF* pSecDF with HUVECs. (A) Effect of *secDF* inactivation in the CHE482 background on adhesion. (B) Growth in invasion medium. The arrow indicates the time point at which extracellular bacteria are lysed. (C) Inoculum dependent cytotoxicity of wild-type strains as determined by the MTT assay. (D) Effect of *secDF* inactivation in the CHE482 background on cytotoxicity as determined by the MTT assay. (E) Effect of *secDF* inactivation in the CHE482 background on invasion. ***, $P < 0.0001$.

Inactivation of *secDF* leads to reduced pathogenicity in an insect infection model

To assess the influence of SecDF on virulence, Newman strains were injected into last instar larval stage *G. mellonella* and survival of the larvae was monitored over time. Resuspension buffer for bacterial cultures was used as a negative control. The dimensions and masses of larvae in one group spanned representative and comparable ranges, as preparatory experiments did not show any correlation concerning larval weight and survival (data not shown). Pathogenicity of Newman Δ*secDF* pEmpty in *G. mellonella* was significantly reduced ($P < 0.001$) in comparison to the wild type Newman pEmpty and the complemented mutant Newman Δ*secDF* pSecDF (Figure 5A). To ensure that the attenuated virulence was not due to growth deficiencies of the mutant, the bacterial burden per larvae was measured after 24, 48 and 72 hours. Variation of CFU per larvae was rather high within the strains, for instance at 24 hours post infection (hpi) the wild type showed a bacterial burden ranging from 1.54 x 10^5 to 4.62 x 10^7 (Figure 5B). However, all three strains were able to multiply in the larvae within a similar range during the first 48 hpi, with CFUs being even higher in the *secDF* mutant after 72 hpi than in the wild type and the complemented mutant. The different peak time points of CFU/larvae in the *secDF* mutant (72 hpi) compared to the wild type or complemented mutant (48 hpi) was not reflected in the survival of *G. mellonella*, further indicating a strongly reduced virulence in Newman Δ*secDF* pEmpty, that was not compensated by increased CFU numbers.

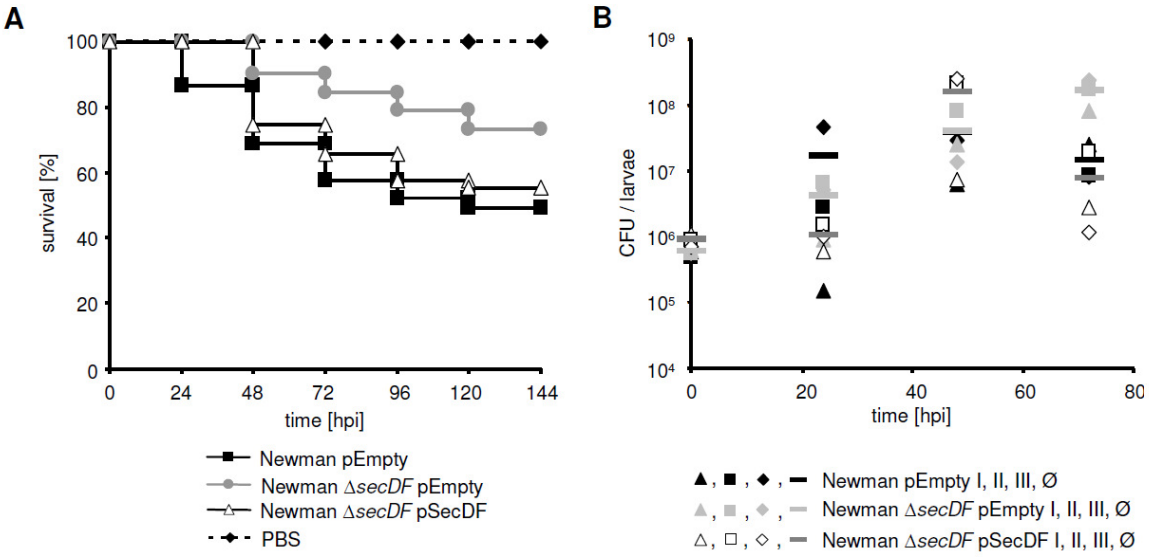

Figure 5. *G. mellonella* virulence assay. (A) Pathogenicity of Newman pEmpty, Newman Δ*secDF* pEmpty and Newman Δ*secDF* pSecDF in *G. mellonella*. Larvae were monitored every 24 hours. PBS was used as negative control. Three independent experiments were pooled and plotted as Kaplan-Meier survival curve, $P < 0.001$. (B) Bacterial burden per (live) larvae was measured in triplicates 24,

48 and 72 h post infection (hpi). The symbols triangle, square, diamond and line correspond to replicates I, II, III and the average, Ø, respectively. The inoculum (CFU/larvae) is shown at time point zero.

Discussion

Secretion of numerous virulence factors such as adhesins, proteases, autolysins and toxins rely on a functional Sec secretion system for the export across the cytoplasmic membrane [43]. So far, most studies of the Sec machinery were performed in the Gram-negative and Gram-positive model organisms *E. coli* and *B. subtilis*, respectively. This study focused on the impact of the auxiliary SecDF protein on virulence in the human pathogen *S. aureus* by analysing the exoproteome of Newman Δ*secDF* in a gel-free approach, followed by *in vitro* and *in vivo* virulence studies.

Our secretomics results were in good agreement with previous data [23]. A relatively high number of cytoplasmic proteins was found in the SN, which has already been observed by other research groups and in different microorganisms [72,73,74,75]. This occurrence has been assigned to cell wall turnover, proteolytic processing, shedding, natural lysis and lysis due to handling [76,77,78]. Nevertheless, we identified several new proteins to be influenced by SecDF. This was confirmed by Western blot analysis and could be complemented for the proteins CHIPS, Eap, FnBPA, LytM and SEA.

Eight out of 11 proteins, identified in both the previously characterized *S. aureus* RN4220 *secG* mutant [7] and Newman Δ*secDF*, showed a similar trend and were found to be reduced in both mutants in comparison to the wild types (Sle1, Geh, Hlb, Hla, HlgB, HlgC, NWMN_1927 and YfnI). Three proteins (IsaA, Spa and SsaA) were found to be increased in the *secG* mutant, but reduced in Newman Δ*secDF*. Different methods, sampling time points and strain backgrounds could have contributed to those divergent findings. Another possibility is that SecDF is required for a subset of proteins deviating from the ones that require SecG. In addition, since they have different functions in the Sec pathway, the absence of SecG or SecDF might lead to only partially overlapping phenotypes.

Two Sec-dependent proteins were found to be increased in Newman Δ*secDF*; the autolysin LytM and the transglycosylase SceD. Kouwen *et al.* had observed, that a *B. subtilis* LipA hyper-producing strain could export the normally Sec-dependent LipA, which also contains a potential Tat RR-SP via both Tat pathways [79]. *S. aureus* LytM and SceD do not contain a potential Tat RR-SP; whether they are secreted by another transport system in Newman Δ*secDF* remains to be determined.

In the *secG* mutant only one Sec-independent protein was identified to be reduced; YfnI/LtaS [7], which was also found reduced in Newman Δ*secDF* (Supplemental Table S1). Additionally, the two Sec-independent proteins Hld and SEA were found to be significantly reduced upon deletion of *secDF*. Hld belongs to the phenol-soluble modulins and has recently

been shown to be exported by the ABC transporter PMT [80]. The fact that the amounts of certain Sec-independent proteins were changed in both the *secG* and the *secDF* mutant, points towards indirect effects caused by the deletion of these Sec translocase constituents. Slightly reduced levels for the regulatory and Hld-encoding mRNA RNAIII have been shown previously in Newman Δ*secDF* [23]. Because RNAIII transcription is regulated by the two-component system accessory gene regulator (*agr*) the reduction of RNAIII levels suggest that *agr* is indirectly affected by *secDF* deletion [23,81]. Clearly, more work is required to confirm this hypothesis and to determine whether other regulatory processes are influenced directly or indirectly by the absence of SecDF.

Secretome analysis showed decreased SpA levels in Newman Δ*secDF* consistent with a previously reported reduction of *spa* transcription [23]. In contrast, previous and present Western blot analysis showed similar levels of full-length SpA in the wild type and Newman Δ*secDF*. A possible explanation for these seemingly contradictory findings could be the reduced amounts of SspA protease in the Newman Δ*secDF* (Supplemental Table S1). SspA is the main player of SpA degradation [82]. Higher SpA and SspA levels in the wild type would lead to more SpA degradation fragments, which can be detected by secretome analysis and increase the total protein amounts attributed to SpA, but would be too small to be detected by Western blot analysis. Whether this is the case still needs to be investigated.

Several well known virulence factors playing a central role during the initial steps of an infection were decreased in Newman Δ*secDF*. These changes led to reduced binding to the two important human host factors fibrinogen and fibronectin *in vitro*. Although the effect of the *secDF* deletion on the adhesion intensity varied between the two strains tested and was smaller in CHE482, we also found significantly reduced adhesion of CHE482 Δ*secDF* to HUVECs. This was accompanied by a significantly decreased invasion and must be attributed to the sum of impaired factors in the mutant and the multitude of additional host matrix factors *S. aureus* can bind to. In this study, the two important invasion factors Eap and FnBPA were found to be reduced [83,84]. While the receptor of Eap is still unknown, the FnBPs bind via the bridging factor fibronectin to the host receptor integrins $\alpha_5\beta_1$, which is sufficient to induce the uptake of staphylococci [83]. Thus, the low cytotoxicity in HUVECs displayed by CHE482 Δ*secDF* must be assumed to be at least partially caused by its defective adhesion and invasion, in addition to a reduced production of toxins. The decreased levels of numerous virulence factors and the reduced cytotoxicity, indicative for virulence in mice and rabbits [38,85,86], suggested the virulence of the *secDF* mutant to be attenuated. We therefore determined the pathogenicity of Newman Δ*secDF* pEmpty in the invertebrate model

host *G. mellonella*, sharing several features of the innate immune response with mammalians [87,88]. A positive correlation has been shown between the pathogenicity of microorganisms in insects and in mice [89,90] and in recent years this model has been increasingly used for assessing the virulence of *S. aureus* [41,91,92,93,94]. As expected, virulence was significantly attenuated in Newman Δ*secDF* pEmtpy. The similar bacterial load per larvae indicated that Newman Δ*secDF* pEmtpy has the ability to multiply as well as the parent strain and that the generally diminished virulence factor expression Newman Δ*secDF* pEmtpy is the cause for its reduced virulence.

Taken together, our data provide new insights on the relevance of SecDF in *S. aureus* pathogenicity. We showed that deletion of *secDF* affects an important part of the extracellular proteome leading to reduced adhesion, invasion and cytotoxicity, as well as reduced virulence in *G. mellonella*. Because both MSSA and MRSA *secDF* mutants are less resistant to well established antibiotics [23], SecDF is an interesting target for the development of novel antimicrobial substances.

Author Contributions

Conceived and designed the experiments: CQ, MMS; Performed the experiments: CQ, KS, BR, MMS; Analyzed the Data: CQ, BR, MMS, KS; Contributed reagents/materials/analysis tools: AZ, BR, BBB; Wrote the paper: CQ, KS, MMS

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Supplemental Table S1. Proteins found in the supernatant of *S. aureus* Newman (wt) and the mutant Newman Δ*secDF* (*secDF*) and their predicted localization.

NWMN	Protein name	Gene	wt I	wt II	wt III	wt IV	*secDF* I	*secDF* II	*secDF* III	*secDF* IV	ø wt	ø *secDF*	*P*	SignalP [1]	Sec SP [2]	No SP(x) [2]	SecretomeP [3]	Localization [4,5]	
			fold change (log 2)																
0002	DNA polymerase III subunit beta	*dnaN*	0	0.16			0.01	-0.13			0.08	-0.06						Cytoplasm	
0014	50S ribosomal protein L9	*rplI*	0	-0.51			-0.61	-0.95			-0.26	-0.78					Yes	Cytoplasm	
0030	Probable tRNA-dihydrouridine synthase	*dus*	0	-0.12			-0.99	-0.32			-0.06	-0.66						Cytoplasm	
0055	Immunoglobulin G-binding protein A	*spa*	0	-0.37	0	-0.29	-3.01	-3.10	-2.40	-2.52	-0.16	-2.76	***	Yes	Yes		Yes	Cell wall	Sortase substrate
0113	Putative aldehyde dehydrogenase	*aldA*	0	0.16	0	-0.24	-0.15	-0.78	-0.46	-0.56	-0.02	-0.49	*					Cytoplasm	
0129	Ornithine aminotransferase 2	*argD*	0	0.05	0	-0.56	-0.34	-0.44	-0.68	-1.10	-0.13	-0.64						Cytoplasm	
0166	Staphylocoagulase	*coa*	0	0.14	0	0.08	-4.33	-3.91	-2.61	-3.26	0.06	-3.53	**	Yes	Yes		Yes	Extracellular	
0172	Putative zinc metalloprotease	*0172*	0	0.10			-0.67	-0.65			0.05	-0.66	*				Yes	Membrane	
0189	2-C-methyl-D-erythritol 4-phosphate cytidylyl-transferase 2	*ispD2*	0	0.17			-0.06	-0.35			0.08	-0.21						Unknown	
0210	Glycyl-glycine endopeptidase	*lytM*	0	0.03	0	-0.35	2.00	2.06	1.72	1.34	-0.08	1.78	***	Yes	Yes		Yes	Extracellular	
0249	5'-nucleotidase, lipoprotein e (P4)	*0249*	0	-0.27	0	0.22	-2.22	-2.16	-1.58	-1.75	-0.01	-1.93	***	Yes	Yes		Yes	Extracellular	
0262	Lipase 2	*geh*	0	-0.18	0	-0.19	-2.93	-2.92	-1.63	-1.75	-0.09	-2.31	**	Yes	Yes		Yes	Extracellular	
0357	30S ribosomal protein S6	*rpsF*	0	-0.14	0	-0.24	-0.36	-0.22	0.20	-0.09	-0.09	-0.12						Cytoplasm	
0371	Alkyl hydroperoxide reductase subunit F	*ahpF*	0	-0.19	0	-0.66	-0.47	-0.70	-0.86	-1.08	-0.21	-0.78	*			x		Cytoplasm	
0372	Alkyl hydroperoxide reductase subunit C	*ahpC*	0	0.13	0	-0.34	-0.05	-0.37	-0.07	-0.39	-0.05	-0.22				x	Yes	Cytoplasm	
0375	Conserved hypothetical protein	*0375*	0	-0.32			-1.06	-1.05			-0.16	-1.06					Yes	Cytoplasm	
0377	Conserved hypothetical protein	*0377*	0	0.00	0	-0.46	-0.59	-0.81	-0.50	-0.52	-0.12	-0.60	*				Yes	Cytoplasm	
0380	Inosine-5'-monophosphate dehydrogenase	*guaB*	0	-0.06	0	-0.01	0.02	-0.05	-0.40	-0.57	-0.02	-0.25				x		Cytoplasm	
0381	GMP synthase [glutamine-hydrolyzing]	*guaA*	0	-0.20			-0.31	-0.63			-0.10	-0.47				x		Cytoplasm	
0429	N-acetylmuramoyl-L-alanine amidase	*sle1*	0	-0.38	0	0.51	-1.45	-0.92	-0.12	-1.05	0.03	-0.89	*	Yes	Yes		Yes	Cell wall, non-cov.	LysM domain [6]
0443	Conserved hypothetical protein	*0443*	0	-0.07			-0.27	-0.39			-0.03	-0.33						Unknown	
0453	Methionyl-tRNA synthetase	*metS*	0	-0.33	0	-0.05	-0.87	-1.20	-0.35	-0.74	-0.09	-0.79	*					Cytoplasm	
0463	Ribose-phosphate pyrophosphokinase	*prs*	0	0.36			0.37	-0.04			0.18	0.17						Cytoplasm	
0464	50S ribosomal protein L25	*rplY*	0	0.07	0	-0.56	0.37	0.19	-0.21	-0.01	-0.12	0.08						Cytoplasm	
0472	Hypoxanthine-guanine phosphoribosyl-transferase	*hpt*	0	0.48	0	-0.65	0.11	0.44	2.31	1.36	-0.04	1.06						Cytoplasm	
0475	Cysteine synthase homolog	*cysK*	0	0.06	0	-0.08	-0.37	-0.45	-0.29	-0.44	-0.01	-0.39	***					Cytoplasm	
0479	Lysyl-tRNA synthetase	*lysS*	0	-0.25			-0.56	-0.42			-0.12	-0.49						Cytoplasm	
0481	Pyridoxal biosynthesis lyase	*pdxS*	0	-0.28	0	-1.02	-0.44	-0.39	-0.32	-0.85	-0.33	-0.50						Cytoplasm	
0482	Glutamine amidotransferase subunit	*pdxT*	0	-0.47			-0.26	-0.18			-0.23	-0.22						Cytoplasm	
0487	ATP-dependent Clp protease, ATP-binding subunit ClpC	*clpC*	0	0.40	0	-0.17	-0.05	0.02	-0.28	-0.44	0.06	-0.19				x		Cytoplasm	
0490	Glutamyl-tRNA synthetase	*gltX*	0	0.61	0	-0.54	0.01	-0.17	-0.44	-0.91	0.02	-0.38				x		Cytoplasm	
0498	Transcription antitermination protein	*nusG*	0	0.13			-0.32	-0.39			0.06	-0.35						Cytoplasm	
0499	50S ribosomal protein L11	*rplK*	0	0.15	0	-0.26	0.21	0.23	0.13	0.31	-0.03	0.22						Cytoplasm	
0500	50S ribosomal protein L1	*rplA*	0	0.23	0	-0.25	0.12	-0.16	0.06	-0.14	0.00	-0.03					Yes	Cytoplasm	
0501	50S ribosomal protein L10	*rplJ*	0	0.06	0	-0.48	0.09	-0.04	0.00	0.04	-0.10	0.02						Cytoplasm	

NWMN	Protein name	Gene	wt I	wt II	wt III	wt IV	*secDF* I	*secDF* II	*secDF* III	*secDF* IV	ø wt	ø *secDF*	*P*	SignalP [1]	Sec SP [2]	No SP(x) [2]	SecretomeP [3]	Localization [4,5]	
			fold change (log 2)																
0502	50S ribosomal protein L7/L12	*rplL*	0	0.06	0	-0.36	0.30	-0.02	-0.07	-0.12	-0.07	0.02						Cytoplasm	
0504	DNA-directed RNA polymerase subunit beta	*rpoB*	0	0.03			-0.30	-0.29			0.01	-0.30	*					Cytoplasm	
0505	DNA-directed RNA polymerase subunit beta'	*rpoC*	0	0.16	0	-0.34	-0.41	-0.49	0.07	-0.48	-0.05	-0.33						Cytoplasm	
0508	30S ribosomal protein S7	*rpsG*	0	-0.38	0	-0.70	0.42	0.36	-0.40	-0.24	-0.27	0.03						Cytoplasm	
0509	Elongation factor G	*fus*	0	0.06	0	-0.38	0.33	0.26	-0.63	-0.51	-0.08	-0.14				x		Cytoplasm	
0510	Elongation factor Tu	*tufA*	0	-0.05	0	-0.21	0.77	0.65	0.67	0.72	-0.07	0.71	***			x		Cytoplasm	
0512	Conserved hypothetical protein	*0512*	0	-0.12			-0.53	-0.89			-0.06	-0.71						Cytoplasm	
0513	Similar to chaperone protein HchA	*hchA*	0	-0.27	0	-0.44	0.05	-0.01	-0.38	-0.39	-0.18	-0.18					Yes	Cytoplasm	
0516	Probable branched-chain-amino-acid amino-transferase	*ilvE*	0	0.11	0	-0.32	0.49	0.02	-0.51	-0.65	-0.05	-0.16						Cytoplasm	
0525	Serine-aspartate repeat-containing protein E	*sdrE*	0	-0.08	0	-0.44	-0.92	-1.00	-0.81	-1.03	-0.13	-0.94	**	Yes	Pot.		Yes	Cell wall	Sortase substrate
0533	3-hexulose-6-phosphate synthase	*0533*	0	-0.32	0	-0.52	0.05	-0.16	0.37	-0.09	-0.21	0.04						Cytoplasm	
0550	Conserved hypothetical protein	*0550*	0	-0.03	0	0.56	0.62	-0.56	0.86	0.71	0.13	0.41						Unknown	
0551	Phosphate acetyltransferase	*pta*	0	0.02	0	-0.39	-0.21	-0.51	-0.53	-0.43	-0.09	-0.42	*			x		Cytoplasm	
0579	Arginyl-tRNA synthetase	*argS*	0	0.31			-1.65	-0.87			0.16	-1.26						Cytoplasm	
0588	Transcriptional regulator	*sarA*	0	0.00			-0.03	-0.26			0.00	-0.15						Cytoplasm	
0655	MarR family regulatory protein	*mgrA*	0	-0.29			0.01	-0.22			-0.14	-0.10					Yes	Cytoplasm	
0687	Glycerol phosphate lipoteichoic acid synthase	*ltaS*	0	0.04	0	-0.15	-0.70	-0.69	-0.69	-0.75	-0.03	-0.71	***				Yes	Membrane	
0721	Ribosomal protein S30EA	*0721*	0	-0.35			-0.67	-0.55			-0.18	-0.61				x		Cytoplasm	
0732	Thioredoxin reductase	*trxB*	0	0.27			-0.06	-0.15			0.14	-0.11				x		Cytoplasm	
0736	ATP-dependent Clp protease, ATP-binding subunit ClpP	*clpP*	0	-0.33			-0.36	-0.69			-0.16	-0.53				x		Cytoplasm	
0741	Glyceraldehyde-3-phosphate dehydrogenase 1	*gapA*	0	0.00	0	-0.14	0.78	0.55	0.43	0.97	-0.03	0.68	**			x		Cytoplasm	
0742	Phosphoglycerate kinase	*pgk*	0	0.05	0	0.04	0.23	0.08	-0.07	-0.39	0.02	-0.04				x		Cytoplasm	
0743	Triosephosphate isomerase	*tpi*	0	-0.18	0	-0.45	0.31	0.24	-0.36	0.19	-0.16	0.09				x		Cytoplasm	
0744	2,3-bisphosphoglycerate-independent phosphoglycerate mutase	*pgm*	0	-0.71			-3.04	-3.31			-0.35	-3.17				x		Cytoplasm	
0745	Enolase	*eno*	0	-0.13	0	-0.14	-0.01	-0.23	-0.38	-0.28	-0.07	-0.23				x		Cytoplasm	MLS
0756	Clumping factor A	*clfA*	0	0.13	0	-0.23	-1.48	-1.55	-1.34	-1.34	-0.02	-1.42	***	Yes	Yes		Yes	Cell wall	Sortase substrate
0758	Extracellular matrix protein	*emp/ssp*	0	-0.04	0	-0.36	-3.18	-3.05	-2.68	-3.00	-0.10	-2.98	***	Yes	Yes		Yes	Extracellular / CW	Can re-bind to CW, non-cov. [7]
0776	Glycine cleavage system H protein	*gcvH*	0	-0.20	0	-0.52	0.10	-0.11	-0.25	-0.42	-0.18	-0.17						Cytoplasm	
0783	CsbD-like superfamily protein	*0783*	0	-0.06	0	-0.29	-0.75	-0.87	-0.14	-0.83	-0.09	-0.65	*				Yes	Unknown	
0800	Hydrolase	*nagD*	0	0.14	0	-0.08	-0.29	-0.68	-0.33	-0.42	0.02	-0.43	**				Yes	Unknown	
0803	D-alanine-D-alanyl carrier protein ligase	*dltA*	0	-0.15			0.02	-0.24			-0.08	-0.11						Cytoplasm	
0805	D-alanyl carrier protein	*dltC*	0	-0.26	0	-0.31	0.11	0.12	0.00	0.48	-0.14	0.18						Cytoplasm	
0824	Putative peptidyl-prolyl cis-trans isomerase	*ppi*	0	-0.24	0	-0.19	0.06	-0.25	0.01	-0.38	-0.11	-0.14					Yes	Cytoplasm	
0833	Glucose-6-phosphate isomerase	*pgi*	0	-0.19	0	-0.13	-0.70	-0.51	-0.26	-0.26	-0.08	-0.43	*			x		Cytoplasm	
0836	Signal peptidase IB	*spsB*	0	-0.51			-0.77	-0.08			-0.26	-0.43					Yes	Membrane	MLS
0839	Fumarylacetacetate hydrolase family protein	*0839*	0	-0.02	0	-0.32	0.11	0.15	-0.15	-0.15	-0.09	-0.01					Yes	Cytoplasm	

NWMN	Protein name	Gene	wt I	wt II	wt III	wt IV	*secDF* I	*secDF* II	*secDF* III	*secDF* IV	ø wt	ø *secDF*	*P*	SignalP [1]	Sec SP [2]	No SP(ˣ) [2]	SecretomeP [3]	Localization [4,5]	
			fold change (log 2)																
0845	Chaperone protein	*clpB*	0	-0.38	0	-0.01	0.20	-0.09	-2.34	-2.73	-0.10	-1.24						Cytoplasm	
0854	3-oxoacyl-[acyl-carrier-protein] synthase 2	*fabF*	0	0.00	0	-0.63	0.44	0.63	-0.27	-0.36	-0.16	0.11						Cytoplasm	
0885	2', 5' RNA ligase	*0885*	0	-0.04	0	-0.09	-0.33	-0.33	-0.44	-0.33	-0.03	-0.36	***					Cytoplasm	
0915	1,4-Dihydroxy-2-naphthoyl-CoA synthase	*menB*	0	0.22	0	-0.51	-0.18	-0.32	-0.27	-0.30	-0.07	-0.27						Cytoplasm	
0917	Cysteine protease	*sspB*	0	-0.02	0	-0.22	-1.38	-1.33	-0.65	-1.14	-0.06	-1.13	**	Yes	Yes		Yes	Extracellular	
0918	V8 protease, glutamyl endopeptidase precursor	*sspA*	0	-0.16			-1.19	-1.64			-0.08	-1.41		Yes	Yes		Yes	Extracellular	
0922	Bifunctional autolysin	*atl*	0	1.21	0	-0.34	1.67	0.91	0.19	0.12	0.22	0.72		Yes	Yes		Yes	Extracellular	
0925	Similar to cell envelope-related transcriptional attenuator	*0925*	0	-0.25	0	-0.54	-1.27	-1.31	-1.28	-1.90	-0.20	-1.44	***	Yes		x	Yes	Membrane	predicted uncleaved SP
0930	Probable quinol oxidase subunit 2	*qoxA*	0	-0.11			-0.88	-0.94			-0.06	-0.91	*				Yes	Membrane	
0932	Bifunctional protein folD	*folD*	0	-0.17			-0.40	-0.18			-0.09	-0.29						Cytoplasm	
0949	Histidine-containing phosphocarrier protein	*ptsH*	0	-0.20	0	-0.38	0.24	-0.01	-0.22	-0.15	-0.14	-0.04				x		Cytoplasm	
0950	Phosphoenolpyruvate-protein phospho-transferase	*ptsI*	0	-0.24	0	-0.46	0.04	0.06	-0.42	-0.73	-0.17	-0.26				x		Cytoplasm	
0959	Pyruvate dehydrogenase E1 component subunit alpha	*phdA*	0	-0.05	0	-0.52	0.05	-0.22	-0.29	-0.63	-0.14	-0.27				x		Cytoplasm	
0960	Pyruvate dehydrogenase E1 component subunit beta	*phdB*	0	-0.07	0	-0.28	0.20	-0.04	0.00	-0.10	-0.09	0.01				x		Cytoplasm	
0961	Dihydrolipoyllysine-residue acetyltransferase component of pyruvate dehydrogenase complex	*pdhC*	0	0.08	0	-0.35	0.02	-0.11	-0.13	-0.37	-0.07	-0.15				x		Cytoplasm	
0962	Dihydrolipoyl dehydrogenase	*pdhD*	0	-0.21	0	0.02	-0.39	-0.56	-0.24	-0.31	-0.05	-0.37	*			x		Cytoplasm	
1019	Putative major head protein Orf47	*1019*	0	0.12			-1.65	-1.53			0.06	-1.59	**					Cytoplasm	
1041	Iron-regulated surface determinant protein A	*isdA*	0	-0.08	0	-0.26	-0.53	-0.64	-0.31	-0.59	-0.08	-0.51	**	Yes	Yes		Yes	Cell wall	Sortase substrate
1057	Thioredoxin	*trxA*	0	-0.11	0	-0.35	0.79	0.56	0.18	-0.01	-0.12	0.38						Cytoplasm	
1067	FPRL1 inhibitory protein / FLIPr	*flr*	0	-0.16	0	-0.31	-4.07	-3.87	-3.04	-3.25	-0.12	-3.56	***	Yes	Yes		Yes	Extracellular	
1069	Fibrinogen-binding protein	*efb/fib*	0	0.27	0	0.09	-1.49	-1.11	-1.38	-1.34	0.09	-1.33	***	Yes	Yes		Yes	Extracellular / CW	Can re-bind to CW, non-cov. [7]
1073	Alpha-hemolysin	*hla*	0	0.02	0	-0.46	-1.16	-1.44	-0.67	-0.97	-0.11	-1.06	**	Yes	Yes		Yes	Extracellular	
1086	Uncharacterized N-acetyltransferase	*1086*	0	0.07			-0.68	-0.54			0.04	-0.61	*					Unknown	
1096	Cell division protein	*ftsZ*	0	-0.19	0	-0.60	-0.29	-0.16	-0.47	-0.24	-0.20	-0.29						Cytoplasm	
1103	Isoleucyl-tRNA synthetase OS	*ileS*	0	0.08	0	-0.19	-0.45	-0.44	-0.12	-0.51	-0.03	-0.38	*			x		Cytoplasm	
1125	Peptide deformylase	*def*	0	-0.60			-0.34	-0.79			-0.30	-0.57						Cytoplasm	
1139	Phosphate acyltransferase	*plsX*	0	1.27			-0.86	0.15			0.63	-0.36						Cytoplasm	
1140	Malonyl CoA-acyl carrier protein transacylase	*fabD*	0	-0.63			-0.15	-0.77			-0.31	-0.46						Cytoplasm	
1141	3-oxoacyl-[acyl-carrier-protein] reductase	*fabG*	0	-0.40	0	0.12	-0.65	-0.83	-0.47	-0.32	-0.07	-0.57	*					Cytoplasm	
1148	30S ribosomal protein S16	*rpsP*	0	-0.01	0	-0.39	0.13	-0.14	-0.42	-0.44	-0.10	-0.22					Yes	Cytoplasm	
1155	Succinyl-CoA ligase [ADP-forming] subunit β	*sucC*	0	0.02	0	-0.38	-0.40	-0.59	-0.56	-0.85	-0.09	-0.60	**					Cytoplasm	
1156	Succinyl-CoA ligase [ADP-forming] subunit α	*sucD*	0	0.27	0	-0.12	-0.35	-0.49	0.12	-0.48	0.04	-0.30				x		Cytoplasm	
1165	GTP-sensing transcriptional pleiotropic repressor	*codY*	0	-0.13	0	0.19	0.08	-0.11	0.53	0.86	0.02	0.34						Cytoplasm	

NWMN	Protein name	Gene	wt I	wt II	wt III	wt IV	*secDF* I	*secDF* II	*secDF* III	*secDF* IV	ø wt	ø *secDF*	*P*	SignalP [1]	Sec SP [2]	No SP(x) [2]	SecretomeP [3]	Localization [4,5]	
			fold change (log 2)																
1166	30S ribosomal protein S2	*rpsB*	0	-0.47	0	-0.22	-0.41	-0.40	-0.48	-0.62	-0.17	-0.48						Cytoplasm	
1167	Elongation factor Ts	*tsf*	0	-0.12	0	-0.45	0.08	-0.07	-0.06	-0.13	-0.14	-0.05				x		Cytoplasm	
1169	Ribosome-recycling factor	*frr*	0	0.06	0	-0.44	0.02	-0.22	0.28	0.04	-0.09	0.03						Cytoplasm	
1178	Translation initiation factor IF-2	*infB*	0	-0.23			-0.07	0.11			-0.11	0.02						Cytoplasm	
1181	Polyribonucleotide nucleotidyltransferase	*ribF*	0	-0.44			-1.25	-1.02			-0.22	-1.13						Cytoplasm	
1182	30S ribosomal protein S15	*rpsO*	0	-0.28			-0.39	-0.50			-0.14	-0.44						Cytoplasm	
1208	Glycerol kinase	*glpK*	0	0.19			-0.58	-0.55			0.09	-0.57						Cytoplasm	
1217	Glutamine synthetase	*glnA*	0	-0.03	0	0.08	0.32	0.16	0.59	0.45	0.01	0.38	*			x		Cytoplasm	
1236	Thermonuclease	*nuc*	0	0.00	0	-0.21	-0.57	-0.63	-0.33	-0.66	-0.05	-0.55	**				Yes	Extracellular	
1246	Catalase	*katA*	0	-0.04	0	-0.42	-0.57	-0.75	-0.79	-0.81	-0.11	-0.73	**			x	Yes	Cytoplasm	
1254	Transketolase	*tkt*	0	-0.12	0	-0.13	-0.13	-0.17	-0.07	-0.26	-0.06	-0.16				x	Yes	Unknown	
1263	Aconitate hydratase	*acnA*	0	-0.21	0	-0.11	-0.25	-0.46	-0.39	-0.19	-0.08	-0.32	*				Yes	Cytoplasm	
1274	Regulatory protein	*msrR*	0	-0.02	0	-0.19	-1.46	-1.00	-1.04	-1.14	-0.05	-1.16	***				Yes	Membrane	
1275	4-oxalocrotonate tautomerase	*1275*	0	0.12			0.01	-0.07			0.06	-0.03						Cytoplasm	
1313	Cold shock protein	*cspA*	0	-0.20	0	-0.44	0.44	0.35	0.62	0.12	-0.16	0.38	*			x	Yes	Cytoplasm	
1332	Probable CtpA-like serine protease	*1332*	0	-0.48			-1.22	-0.89			-0.24	-1.05					Yes	Membrane	
1333	Phosphotransferase system glucose-specific IIA component	*crr*	0	-0.46			-0.01	-0.32			-0.23	-0.17						Cytoplasm	
1339	Conserved hypothetical protein	*1339*	0	0.32			-0.36	-0.46			0.16	-0.41						Cytoplasm	
1365	Asparaginyl-tRNA synthetase	*asnS*	0	-0.10			-0.55	-0.59			-0.05	-0.57	*					Cytoplasm	
1378	Nucleoside diphosphate kinase	*ndk*	0	-0.05			-0.53	-0.61			-0.02	-0.57	*					Cytoplasm	
1382	DNA-binding protein HU	*hup*	0	0.10	0	-0.38	0.48	0.24	0.48	0.39	-0.07	0.40	*					Cytoplasm	
1385	30S ribosomal protein S1	*rpsA*	0	-0.09	0	-0.37	-0.42	-0.55	-0.15	-0.33	-0.11	-0.36				x		Cytoplasm	
1386	Cytidylate kinase	*cmk*	0	0.34			0.86	0.17			0.17	0.52						Cytoplasm	
1389	Elastin-binding protein	*ebpS*			0	-0.50			-1.13	-1.23	-0.25	-1.18				x	Yes	Membrane	MLS
1417	6-phosphogluconate dehydrogenase, decarboxylating	*gnd*	0	0.00			0.24	-0.10			0.00	0.07				x		Cytoplasm	
1456	Superoxide dismutase [Mn/Fe]	*sodA*	0	0.26	0	-0.48	-0.26	-0.24	-0.21	-0.43	-0.06	-0.28				x	Yes	Extracellular	No SP
1468	Glycyl-tRNA synthetase	*glyS*	0	0.07	0	-0.07	0.30	0.06	-0.14	-0.08	0.00	0.04						Cytoplasm	
1483	Chaperone protein	*dnaK*	0	0.02	0	-0.40	0.12	0.02	0.14	0.07	-0.10	0.09				x	Yes	Cytoplasm	
1484	Hsp-70 cofactor	*grpE*	0	0.08	0	-0.43	-0.51	-0.57	-0.09	-0.35	-0.09	-0.38				x	Yes	Cytoplasm	
1488	30S ribosomal protein S20	*rpsT*	0	-0.37			-0.59	-0.95			-0.19	-0.77					Yes	Cytoplasm	
1516	Conserved hypothetical protein	*1516*	0	0.00			0.14	-0.02			0.00	0.06						Cytoplasm	
1547	50S ribosomal protein L27	*rpmA*	0	0.14	0	-0.53	-0.02	-0.10	-0.42	-0.47	-0.10	-0.25					Yes	Cytoplasm	
1549	50S ribosomal protein L21	*rplU*	0	0.14	0	-0.97	-0.77	-0.36	-0.55	-0.92	-0.21	-0.65						Cytoplasm	
1558	Valyl-tRNA synthetase	*valS*	0	0.35			0.19	-0.13			0.18	0.03						Cytoplasm	
1569	Trigger factor	*tig*	0	-0.13	0	-0.21	-0.03	-0.23	-0.40	-0.40	-0.09	-0.26				x		Cytoplasm	
1574	Translation initiation factor IF-3	*infC*	0	-0.23			-1.13	-0.50			-0.12	-0.81						Cytoplasm	
1576	Threonyl-tRNA synthetase	*thrS*	0	0.19			0.03	-0.48			0.09	-0.23						Cytoplasm	
1587	Isocitrate dehydrogenase [NADP]	*citC*	0	-0.11	0	-0.35	-0.51	-0.71	-0.49	-1.00	-0.11	-0.68	**					Cytoplasm	

NWMN	Protein name	Gene	wt I	wt II	wt III	wt IV	*secDF* I	*secDF* II	*secDF* III	*secDF* IV	ø wt	ø *secDF*	*P*	SignalP [1]	Sec SP [2]	No SP(x) [2]	SecretomeP [3]	Localization [4,5]	
			fold change (log 2)																
1592	Pyruvate kinase	*pyk*	0	-0.32			-0.72	-0.60			-0.16	-0.66						Cytoplasm	
1593	6-phosphofructokinase	*pfk*	0	-0.14	0	-0.35	-0.56	-0.23	-0.40	-0.24	-0.12	-0.36						Cytoplasm	
1601	Metal-dependent hydrolase	*1601*	0	0.93			0.81	-0.03			0.46	0.39						Cytoplasm	
1604	Universal stress protein family protein	*1604*	0	-0.07	0	-0.40	-0.19	-0.25	-0.43	-0.31	-0.12	-0.29				x		Cytoplasm	
1609	Probable thiol peroxidase	*thil*	0	-0.04	0	-0.09	-0.25	-0.23	0.24	-0.32	-0.03	-0.14						Cytoplasm	
1613	30S ribosomal protein S4	*rpsd*	0	0.13			0.05	-0.09			0.07	-0.02						Cytoplasm	
1625	Formate--tetrahydrofolate ligase	*fhs*	0	0.25	0	-0.39	-0.21	-0.25	-0.18	-0.45	-0.04	-0.27				x		Cytoplasm	
1643	D-alanine aminotransferase	*dat*	0	-0.28			0.29	0.44			-0.14	0.36						Cytoplasm	
1681	Phosphoenolpyruvate carboxykinase [ATP]	*pckA*	0	-0.11			-0.75	-0.85			-0.05	-0.80	**				Yes	Cytoplasm	
1726	Signal transduction protein TRAP (RNAIII-activating protein TRAP)	*traP*	0	-0.08			-0.18	-0.56			-0.04	-0.37				x	Yes	Cytoplasm	
1733	Foldase protein	*prsA*	0	0.01	0	-0.31	-0.49	-0.49	-0.66	-0.82	-0.08	-0.62	**	Yes			Yes	Lipoprotein	
1737	Conserved hypothetical protein	*1737*	0	0.23	0	-0.40	0.83	0.71	-0.14	-0.03	-0.04	0.34						Cytoplasm	
1767	ThiJ/Pfpl family protein	*1767*	0	-0.19	0	-0.58	-0.36	-0.68	-0.56	-0.71	-0.19	-0.58						Cytoplasm	
1831	Ferritin	*ftnA*	0	0.31	0	-0.25	-0.20	-0.49	-0.36	-0.22	0.01	-0.31						Cytoplasm	
1837	Aspartyl/glutamyl-tRNA(Asn/Gln) amidotransferase subunit B	*gatB*	0	-0.37	0	-0.74	-0.23	-0.56	-0.05	-0.24	-0.28	-0.27						Cytoplasm	
1838	Glutamyl-tRNA(Gln) amidotransferase subunit A	*gatA*	0	0.16			0.62	0.34			0.08	0.48					Yes	Cytoplasm	
1839	Aspartyl/glutamyl-tRNA(Asn/Gln) amidotransferase subunit C	*gatC*	0	0.23	0	0.03	-0.40	-0.51	-0.55	-0.62	0.06	-0.52	***				Yes	Cytoplasm	
1847	Staphopain thiol protease	*scpA*	0	-0.23			-2.14	-2.22			-0.12	-2.18	*	Yes	Yes		Yes	Extracellular	
1857	Probable manganese-dependent inorganic pyrophosphatase	*ppaC*	0	-0.07	0	-0.03	-1.00	-0.38	-2.13	-2.27	-0.03	-1.45						Cytoplasm	
1859	Conserved hypothetical protein	*1859*	0	-0.77			-0.91	-1.13			-0.38	-1.02				x		Cytoplasm	
1872	65 kDa membrane protein	*eap/map*	0	-0.42	0	-0.27	-1.79	-1.61	-2.06	-2.21	-0.17	-1.92	***	Yes	Yes		Yes	Extracellular / CW	Can re-bind to CW, non-cov. [8]
1876	Staphylococcal complement inhibitor	*scn*	0	0.20	0	-0.05	-1.65	-1.66	-3.18	-3.46	0.04	-2.49	*	Yes	Yes		Yes	Extracellular	
1877	Chemotaxis inhibitory protein	*chp*	0	0.47	0	0.63	-2.52	-2.29	-1.25	-1.62	0.28	-1.92	**	Yes	Yes		Yes	Extracellular	
1880	Staphylokinase	*sak*	0	0.08	0	-0.48	-0.87	-0.87	-0.79	-0.82	-0.10	-0.84	**	Yes	Yes		Yes	Extracellular	
1883	Enterotoxin type A	*sea*	0	0.05	0	-0.08	-1.95	-1.89	-1.29	-1.40	-0.01	-1.63	**	Yes			Yes	Extracellular	
1927	Leukocidin/hemolysin toxin family F subunit	*lukF*	0	0.12	0	-0.21	-0.42	-0.54	-1.09	-0.87	-0.02	-0.73	*	Yes	Yes		Yes	Extracellular	
1928	Leukocidin/hemolysin toxin family S subunit	*lukS*	0	-0.03	0	-0.31	-1.42	-1.70	-1.16	-1.14	-0.09	-1.36	***	Yes	Yes		Yes	Extracellular	
1937	Chaperonin 60 kDa sunbunit	*groEL*	0	0.03	0	-0.61	-0.28	-0.51	-0.41	-0.47	-0.15	-0.42				x		Cytoplasm	
1938	10 kDa chaperonin	*groES*			0	-0.79			-0.41	-0.69	-0.40	-0.55				x		Cytoplasm	
1972	Anti-sigma-B factor antagonist	*rsbV*	0	-0.15	0	-0.11	-0.79	-0.87	-0.29	-0.09	-0.07	-0.51						Cytoplasm	
1999	Probable transglycosylase	*sceD*	0	0.09	0	-0.49	1.81	1.81	1.60	1.38	-0.10	1.65	***	Yes	Yes		Yes	Extracellular	Can ionically bind to cell wall [9]
2004	UDP-N-acetylglucosamine 1-carboxyvinyltransferase 1	*murA1*	0	1.01			-1.90	-0.17			0.51	-1.03						Cytoplasm	
2007	ATP synthase subunit beta	*atpD*	0	-0.18			-0.47	-0.74			-0.09	-0.60						Cytoplasm	

NWMN	Protein name	Gene	wt I	wt II	wt III	wt IV	*secDF* I	*secDF* II	*secDF* III	*secDF* IV	ø wt	ø *secDF*	*P*	SignalP [1]	Sec SP [2]	No SP(x) [2]	SecretomeP [3]	Localization [4,5]	
			fold change (log 2)																
2009	ATP synthase subunit alpha	*atpA*	0	-0.08			0.08	-0.30			-0.04	-0.11						Cytoplasm	
2016	Uracil phosphoribosyltransferase	*upp*	0	-0.41			-0.09	-0.14			-0.21	-0.11						Cytoplasm	
2024	50S ribosomal protein L31 type B	*rpmE*	0	-0.08	0	-0.36	-0.25	-0.48	-0.25	-0.58	-0.11	-0.39					Yes	Cytoplasm	
2026	Putative aldehyde dehydrogenase	*2026*	0	-0.21			-0.29	-0.55			-0.11	-0.42						Cytoplasm	
2029	Fructose-bisphosphate aldolase	*fbaA*	0	-0.23	0	-0.24	0.10	-0.06	-0.27	-0.18	-0.12	-0.10				x		Cytoplasm	
2031	CTP synthase	*pyrG*	0	0.12			-0.28	0.52			0.06	0.12						Cytoplasm	
2032	Probable DNA-directed RNA polymerase subunit delta	*rpoE*	0	-0.11			-0.06	-0.33			-0.06	-0.20					Yes	Cytoplasm	
2041	Deoxyribose-phosphate aldolase 1	*deoC*	0	-0.43			-0.63	-0.47			-0.21	-0.55						Cytoplasm	
2056	Glucosamine--fructose-6-phosphate aminotransferase [isomerizing]	*glmS*	0	0.04			0.51	-0.30			0.02	0.11						Cytoplasm	
2062	Phosphoglucosamine mutase	*glmM*	0	0.10			0.07	-0.11			0.05	-0.02						Cytoplasm	
2074	Conserved hypothetical protein	*2074*	0	0.13			-0.33	-0.36			0.06	-0.34				x	Yes	Unknown	
2086	Alkaline shock protein 23	*asp23*	0	0.06	0	-0.18	-0.55	-0.97	-0.65	-0.50	-0.03	-0.67	**			x	Yes	Unknown	
2119	30S ribosomal protein S9	*rpsI*			0	-0.66			-0.30	-0.95	-0.33	-0.63						Cytoplasm	
2120	50S ribosomal protein L13	*rplM*	0	-0.06	0	-0.51	-0.33	-0.41	-0.49	-0.56	-0.14	-0.45				x	Yes	Cytoplasm	
2125	50S ribosomal protein L17	*rplQ*	0	0.02	0	-0.24	0.04	-0.06	-0.19	-0.34	-0.06	-0.14						Cytoplasm	
2126	DNA-directed RNA polymerase subunit alpha	*rpoA*	0	-0.02			-0.14	-0.06			-0.01	-0.10						Cytoplasm	
2127	30S ribosomal protein S11	*rpsK*	0	0.11			-0.23	-0.29			0.06	-0.26					Yes	Cytoplasm	
2128	30S ribosomal protein S13	*rpsM*			0	-0.06			0.36	0.54	-0.03	0.45						Cytoplasm	
2130	Translation initiation factor IF-1	*infA*	0	-0.64	0	-0.10	0.52	0.35	0.88	0.88	-0.18	0.66	**					Cytoplasm	
2131	Adenylate kinase	*adk*	0	-0.02	0	-0.31	0.24	0.05	-0.06	-0.33	-0.08	-0.03						Cytoplasm	
2133	50S ribosomal protein L15	*rplO*	0	-0.01	0	-0.40	-0.29	-0.37	-1.00	-0.81	-0.10	-0.62	*					Cytoplasm	
2134	50S ribosomal protein L30	*rpmD*	0	0.02			-0.02	-0.13			0.01	-0.08					Yes	Cytoplasm	
2135	30S ribosomal protein S5	*rpsE*	0	0.02	0	-0.77	-0.07	-0.23	0.27	0.01	-0.19	-0.01						Cytoplasm	
2137	50S ribosomal protein L6	*rplF*	0	0.00	0	-0.29	0.19	0.13	0.09	0.17	-0.07	0.15					Yes	Cytoplasm	
2138	30S ribosomal protein S8	*rpsH*	0	0.22	0	-0.62	-0.03	0.09	-0.26	-0.20	-0.10	-0.10						Cytoplasm	
2140	50S ribosomal protein L5	*rplE*	0	0.05	0	-0.53	-0.04	-0.05	-0.42	-0.05	-0.12	-0.14						Cytoplasm	
2141	50S ribosomal protein L24	*rplX*	0	0.20			0.27	0.09			0.10	0.18					Yes	Cytoplasm	
2143	30S ribosomal protein S17	*rpsQ*	0	0.07			0.05	0.14			0.04	0.09					Yes	Cytoplasm	
2144	50S ribosomal protein L29	*rpmC*	0	0.05	0	-0.44	-0.25	-0.24	0.07	-0.08	-0.10	-0.13						Cytoplasm	
2145	50S ribosomal protein L16	*rplP*	0	-0.51			-0.98	-0.79			-0.25	-0.88						Cytoplasm	
2146	30S ribosomal protein S3	*rpsC*	0	0.08			0.05	-0.15			0.04	-0.05						Cytoplasm	
2147	50S ribosomal protein L22	*rplV*	0	0.13	0	-0.65	-0.73	-0.40	-0.28	-0.42	-0.13	-0.46						Cytoplasm	
2148	30S ribosomal protein S19	*rpsS*	0	0.13	0	-0.28	-0.46	-0.48	-0.26	-0.53	-0.04	-0.43	*					Cytoplasm	
2149	50S ribosomal protein L2	*rplB*	0	-0.19	0	-0.21	0.05	-0.05	-0.22	-0.31	-0.10	-0.13						Cytoplasm	
2150	50S ribosomal protein L23	*rplW*	0	0.21			0.66	0.42			0.11	0.54						Cytoplasm	
2151	50S ribosomal protein L4	*rplD*	0	-0.93			-0.60	-1.20			-0.47	-0.90						Cytoplasm	
2199	Staphylococcal secretory antigen	*ssaA*	0	0.05	0	-0.06	-1.95	-1.80	-1.75	-1.55	0.00	-1.76	***	Yes	Yes		Yes	Extracellular	Can ionically bind to cell wall [9]

NWMN	Protein name	Gene	wt I	wt II	wt III	wt IV	*secDF* I	*secDF* II	*secDF* III	*secDF* IV	ø wt	ø *secDF*	*P*	SignalP [1]	Sec SP [2]	No SP(ˣ) [2]	SecretomeP [3]	Localization [4,5]	
			fold change (log 2)																
2205	Putative 2-hydroxyacid dehydrogenase	*2205*	0	-0.50	0	-0.60	-0.12	-0.43	-0.25	-0.66	-0.27	-0.36						Cytoplasm	
2270	Conserved hypothetical protein	*2270*	0	-0.24	0	0.04	-0.47	-1.03	-0.65	-1.06	-0.05	-0.80	**	Yes			Yes	Lipoprotein	MLS
2317	Immunoglobulin-binding protein	*sbi*	0	-0.03	0	-0.16	-1.33	-1.23	-1.20	-1.22	-0.05	-1.25	***	Yes	Yes		Yes	Extracellular	
2318	Gamma-hemolysin component A	*hlgA*	0	-0.03	0	-0.33	-0.09	-0.23	-0.67	-0.87	-0.09	-0.46		Yes	Yes		Yes	Extracellular	
2319	Gamma-hemolysin component C	*hlgC*	0	0.08	0	-0.26	-0.32	-0.88	-1.00	-0.99	-0.04	-0.79	*	Yes	Yes		Yes	Extracellular	
2320	Gamma-hemolysin component B	*hlgB*	0	-0.20	0	-0.23	-0.30	-0.58	-0.50	-0.78	-0.11	-0.54	*	Yes	Yes		Yes	Extracellular	
2399	Fibronectin-binding protein A	*fnbA*	0	-0.08	0	-0.07	-2.01	-1.92	-2.78	-2.78	-0.04	-2.37	**	Yes	Yes		Yes	Cell wall	Sortase substrate[1]
2448	ATP-dependent Clp protease, ATP-binding subunit	*clpL*	0	0.11			-0.71	-0.88			0.05	-0.80	*					Cytoplasm	MLS
2454	1-pyrroline-5-carboxylate dehydrogenase	*rocA*	0	-0.21	0	-0.58	-0.67	-0.81	-0.76	-0.84	-0.20	-0.77	*			x		Cytoplasm	
2467	O-acetyltransferase	*oatA*	0	0.35	0	-0.23	-1.07	-1.00	-1.13	-1.32	0.03	-1.13	***				Yes	Membrane	
2469	Probable transglycosylase	*isaA*	0	-0.11	0	-0.04	-1.83	-1.87	-1.43	-1.35	-0.04	-1.62	**	Yes	Yes		Yes	Extracellular	Can ionically bind to cell wall [9]
2480	Alpha/beta hydrolase fold family protein	*2480*	0	-0.04			-0.52	-0.22			-0.02	-0.37					Yes	Cytoplasm	
2503	Fructose-bisphosphate aldolase class 1	*fda*	0	-0.03	0	-0.39	0.37	0.49	0.24	0.10	-0.10	0.30	*			x		Cytoplasm	
2504	Probable malate:quinone oxidoreductase 2	*mqo2*	0	0.31			-0.52	-0.54			0.15	-0.53						Cytoplasm	MLS
2529	Clumping factor B	*clfB*	0	-0.17	0	-0.30	-2.58	-2.64	-1.55	-1.80	-0.12	-2.15	**	yes	Yes		yes	Cell wall	Sortase substrate
2537	Immunodominant staphylococcal antigen B	*isaB*	0	-0.05	0	-0.07	-0.60	-0.56	-1.09	-1.40	-0.03	-0.91	*	yes	Yes		yes	Extracellular	
2569	Lipase 1	*lip*	0	0.27	0	-0.15	-0.68	-0.80	-0.54	-0.69	0.03	-0.67	**	yes	Yes		yes	Extracellular	
2624	Delta-hemolysin	*hld*	0	-0.78	0	-1.09	-2.17	-2.12	-1.95	-2.12	-0.47	-2.09	**					Extracellular	No SP

(ˣ) Extracellular, without known signal peptide; MLS, multiple localization sites; [1], FnBPA is truncated due to a point mutation leading to a stop codon. Thus it lacks the sortase motif LPXTG and is entirely secreted [10]; *, $P < 0.05$; **, $P < 0.01$; *** $P < 0.001$.

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4 Comments and Outlook

The ubiquitous RND superfamily has diverse biological functions, ranging from sterol homeostasis and multidrug export to the morphogen receptor Ptc, which plays a role in *Drosophila melanogaster* development (137, 278). This is the first comprehensive study of the three RND proteins SecDF, SA2056 and SA2339 in the Gram-positive bacterium *S. aureus*.

In *E. coli*, SecDF is postulated to be an accessory protein of the Sec machinery enhancing protein export, in particular at late stages of protein secretion. Deletion of *secDF* in *S. aureus* caused a very complex phenotype, affecting even Sec-independent proteins. As expected, protein secretion was not entirely interrupted when SecDF was missing in *S. aureus*, but the majority of Sec-dependent virulence factors were in some way affected, leading to a decreased virulence in an insect animal model. Considering that SecDF shows a high potential as a target for antimicrobial substances, a next crucial step would be to confirm the importance of SecDF for virulence in a murine infection model and screening for inhibitors of SecDF.

Since membrane proteins are also targeted to the Sec machinery, the insertion of important TCS sensor histidine-kinases into the membrane could be disturbed by the absence of SecDF, leading to an inefficient signalling system as was indicated by the slightly reduced transcription of *agr*. This could be further analyzed by quantitative proteomics of the membrane subcellular fractions and by transcription and expression analysis of major TCS.

Depending on the microorganism different localization patterns were found for the Sec machinery. In *B. subtilis*, SecA and SecY were shown to localize in clusters that were organized in a spiral-like structure across the cell (34). In contrast, SecA of *Streptococcus pyogenes* was restricted to one discrete patch called the ExPortal (235, 236). This Sec translocase containing microdomain is in remote distance from both cell poles and is proposed to serve as a compartment for posttranslocational protein folding. No SecDF homologue was found in *S. pyogenes*, nevertheless, it would be relevant to see where SecDF localizes in *S. aureus*; over the entire cell membrane or in distinct patches.

Another gene of interest is *yajC* lying downstream of *secDF*. In *E. coli*, *yajC* is co-transcribed with *secD* and *secF* (225). Furthermore, YajC forms a heterotrimeric complex with SecDF and was found to interact with AcrB (274). However, the function of YajC is still unknown. Northern blot analysis in *S. aureus* revealed *yajC* to be transcribed alone and with the downstream lying gene *tgt* encoding a queuine tRNA-ribosyltransferase, but not with *secDF*

(unpublished data). This is in accordance with the predicted promoters upstream of *yajC* and *tgt*, and with a Rho-independent terminator situated between *yajC* and *secDF*. A *yajC* single and a *yajC-secDF* double mutant could provide more insights into a possible role of YajC in the Sec pathway. Another interesting aspect would be the identification of SecDF interaction partners in *S. aureus* and to verify the interaction partners found for *E. coli* SecDF (YajC, SecYEG and YidC) with the *S. aureus* SecDF.

The roles of SA2056 and the MmpL-like protein SA2339 still have to be elucidated, as for both mutants no major phenotype was obtained under the conditions tested. The strain background can play a crucial role for studying gene function, as reported for the expression of the extracytoplasmic-function σ-factor (ECF) σ^S, which was shown to be differently expressed depending on the strain (177). Hence, it should be reconsidered to construct the *rnd* knock-out mutants in other strain backgrounds. In addition, functional redundancy of the *S. aureus* RND proteins should be excluded by constructing RND double mutants and a RND triple mutant containing the inducible *rnd* genes *in trans*.

SA2056 and SA2339 seem to have very high substrate specificity as testing several different compounds did not reveal any effects; further screening of possible allocrites (compounds to be transported) or interaction partners could be helpful to determine their role in *S. aureus*.

Phenotype analysis of *sa2056* and *sa2339* are primarily based on *in vitro* assays, hence it would be of major interest to analyse their relevance *in vivo*. To keep animal models to a minimum, this could first be performed in a whole blood killing assay and by using an invertebrate infection model.

The interaction of SA2056 with FemB and other proteins involved in PG synthesis indicates an auxiliary role of SA2056 in cell wall synthesis. SA2056 was also shown to interact with itself suggesting formation of a trimer as shown for the multidrug efflux pump AcrB in *E. coli* (186). Thus, determining the crystal structure of SA2056 might reveal more clues regarding its purpose.

Genome sequencing of an *in vitro* generated ceftobiprole resistant *mecA*-negative strain COL revealed five single-nucleotide polymorphisms (SNPs) in three different genes; the carboxypeptidase *pbp4*, the cyclic-di-AMP phosphodiesterase *gdpP* and *sa2056* (11). Ceftobiprole is a 5th generation cephalosporin with binding activity against all PBPs including PBP2a (145). Interestingly, PBP4 and GdpP both directly or indirectly have an influence on the cross-linking of PG and β-lactam resistance (99, 117, 156, 175, 226, 256), further supporting the hypothesis of SA2056 playing a role in cell wall synthesis. Preliminary tests showed that the *sa2056* mutant in an MRSA background was only slightly more susceptible

towards ceftobiprole than the wild type (unpublished data). These results are in need of complementation and further investigations. A possible indicator for a disturbed cell wall biosynthesis is increased basal expression of the cell wall stress stimulon (CWSS) (140, 151, 285). The CWSS is activated by the autoregulated TCS VraSR and can be measured by a promoter-luciferase fusion of the promoter of the *vraSR*-operon (66). Initial tests showed a slightly increased CWSS for the *secDF* mutant (unpublished data), which is in accordance with the aberrant cell separation. CWSS examinations will be extended for the *sa2056* and the *sa2339* mutant.

In this study three *S. aureus* RND proteins were analyzed and two, SA2056 and SecDF were characterized in more detail. The importance of this protein family was shown by the deletion of *secDF* revealing a major impact on resistance to well-known antibiotics and on *S. aureus* virulence. Thus, it is of great value to gain more knowledge of the different functions of these transporter proteins and to find their possible substrates.

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6 Appendix

6.1 CV

Publications

Quiblier C, Zinkernagel A, Schuepbach R, Berger-Bächi B, Senn M (2011) Contribution of SecDF to *Staphylococcus aureus* resistance and expression of virulence factors. BMC Microbiol 11: 72.

Quiblier C, Luczak-Kadlubowska A, Holdener E, Alborn D, Schneider T, et al. (2013) The *Staphylococcus aureus* membrane protein SA2056 interacts with peptidoglycan synthesis enzymes. Antibiotics 2: 11-27.

Quiblier C, Seidl K, Roschitzki B, Zinkernagel AS, Berger-Bächi B, et al. (2013) Secretome analysis defines the major role of SecDF in *Staphylococcus aureus* virulence. PLoS ONE 8: e63513 EP -.

Conferences

Quiblier C, Senn MM, Berger-Bächi B. Determination of the contribution of SecDF to virulence factor production and resistance in *Staphylococcus aureus*. Staphylococcal-Cell Wall Meeting, Tübingen, Germany, 07.05.2010. Poster presentation.

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Quiblier C, Berger-Bächi B, Senn MM. Determination of the contribution of SecDF to virulence and resistance in *Staphylococcus aureus*. International Symposium on Staphylococci and Staphylococcal Infections (ISSS), Bath, United Kingdom, 06.09-09.09.2010. Poster Presentation.

Quiblier C, Berger-Bächi B, Senn MM. Contribution of SecDF to adhesion and virulence in *Staphylococcus aureus*. 10 Years Infection Biology Symposium Biozentrum Basel, Basel, Switzerland, 16.09.2011. Poster presentation.

Quiblier C, Berger-Bächi B, Senn MM. Secretome analysis of a *Staphylococcus aureus secDF* deletion mutant. Joint Annual Meeting of the Swiss Society for Infectious Diseases, the Swiss Society for Hospital Hygiene, the Swiss Society for Microbiology and the Swiss Society of Tropical Medicine and Parasitology, St. Gallen, Switzerland, 21.06.-22.06.2012. Poster presentation.

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